Pectinatus and *Megasphaera*

RNA Based Novel Detection Method

Ashtavinayak Paradh

A thesis submitted for the degree of

Doctor of Philosophy

International Centre for Brewing and Distilling

School of Life Sciences

Heriot Watt University

Edinburgh, UK

October 2013

ABSTRACT

The occurrence of beer spoilage bacteria belonging to the genera *Pectinatus* and *Megasphaera* in ten major UK breweries was investigated. The sampling points were selected from fermentation areas, beer conditioning areas and beer bottling and canning sites. Multiplex PCR methodology was used for detection of three *Pectinatus* and three *Megasphaera* species using species specific primers. The presence of six *Lactobacillus* and three beer spoilage *Pediococcu*s species were also examined. Overall, 117 samples were analysed from ten breweries; six samples were positive for the presence of *Pectinatus* species and three samples were positive for the presence of *Megasphaera* species, while 34 samples were positive for the presence of *Lactobacillus* species and 23 samples were positive for *Pediococcus* species. *Lactobacillus* and *Pediococcus* species appeared to be the major potential spoilage microorganisms. Although none of the actual beer samples were found to be positive for *Pectinatus* and *Megasphaera* species, their occurrence in aerobic brewery environments indicates sanitation problems and revealed the presence of highly established biofilms in some breweries.

The morphological and physiological characterisation of the presumed *Pectinatus* and *Megasphaera* isolates along with NCBI nucleotide blast search assignments and maximum parsimony phylogenetic analysis showed good agreement on their identity. All the *Pectinatus* and *Megasphaera* isolates showed ability to spoil low alcohol beer, and the presence of these microorganisms in the brewery environments could be a potential threat to low alcohol and unpasteurised beer.

Novel implementation of Hybridisation Protection Assay for detection of beer spoilage microorganisms, *Pectinatus* and *Megasphaera* was demonstrated. DNA probes specific for 16S ribosomal RNA were utilised for the detection of beer spoilage bacteria of genera *Pectinatus* and *Megasphaera* using a Hybridization Protection Assay. All the probes were modified during synthesis by inserting an amino linker arm at the 5′ end during synthesis and also internally modified by replacing thymidine base with amine modified thymidine base; synthesised probes were labelled with (AE) and purified using reverse phase HPLC. The designed internally AE-labelled probes were able to detect target RNA within the range of 0.016-0.0032 pmol. All the designed probes showed high specificity towards target RNA and could detect bacterial contamination within the range of ca. $5x10^2$ – $1x10^3$ CFU using HPA assay. The developed assay was also compatible with MRS, NBB and SMMP beer enrichment media.

ACADEMIC REGISTRY
Research Thesis Submission

Name*:*	ASHTAVINAYAK PARADH		
School/PGI:	ICBD, LIFE SCIENCES		
Version: *(i.e. First, Resubmission, Final)*	FINAL	Degree Sought (Award **and** Subject area)	PhD - Brewing and Distilling

Declaration

In accordance with the appropriate regulations I hereby submit my thesis and I declare that:

1) the thesis embodies the results of my own work and has been composed by myself
2) where appropriate, I have made acknowledgement of the work of others and have made reference to work carried out in collaboration with other persons
3) the thesis is the correct version of the thesis for submission and is the same version as any electronic versions submitted*.
4) my thesis for the award referred to, deposited in the Heriot-Watt University Library, should be made available for loan or photocopying and be available via the Institutional Repository, subject to such conditions as the Librarian may require
5) I understand that as a student of the University I am required to abide by the Regulations of the University and to conform to its discipline.

* *Please note that it is the responsibility of the candidate to ensure that the correct version of the thesis is submitted.*

Signature of Candidate*:*		Date:	17-10-2013

Submission

Submitted By *(name in capitals):*	
Signature of Individual Submitting:	
Date Submitted:	

For Completion in the Student Service Centre (SSC)

Received in the SSC by *(name in capitals):*			
Method of Submission *(Handed in to SSC; posted through internal/external mail):*			
*E-thesis Submitted (**mandatory for final theses)***			
Signature:		Date:	

To my family and friends.

ACKNOWLEDGEMENTS

This research project was carried out at The International Centre for Brewing and Distilling, School of Life Sciences, Heriot Watt University, Edinburgh, during the period 2009-2012, funded by the Institute of Brewing and Distilling and the School of Life Sciences, Heriot Watt University, Edinburgh. This financial support was greatly appreciated.

In the beginning, I would like to thank my supervisors Dr. Annie Hill and Dr. Wilfred Mitchell for their assistance, advice, patience and expertise throughout the course of this project. This project would not have been completed without their endless support and encouragement.

I would also like to thank Professor Paul Hughes, Professor David Quain, Dr. Peter Morris and Dr. Derek Jamieson for their interest and valuable suggestions to improve experiments carried out during this study. My special thanks are due to Professor Michael Schweizer for allowing me to use his laboratory for chemiluminescence experiments.

I express my sincere gratitude to all the Brewers and Microbiologists from the ten major breweries of the UK for providing me with excellent advice and technical expertise to carry out a survey during the first year of this research. I am also thankful to Tadhg O'Sullivan from Heineken Brewery, Netherlands for providing pure bacterial cultures.

I sincerely thank Jim McKinlay, Graham McKernan and James Buchanan for helping me with analytical procedures, I am also grateful to Paul Cyphus, Gina Shepherd and Vicky Goodfellow for their invaluable help in microbiological experiments.

I sincerely thank my colleagues in the laboratory over the years. My special thanks are due to Dr. Mohammed Abass, Dr. John Tinsley, Calum Robb, Majde Estoni, Brahim, Dr. Naief Almakeshah, Dr. Ali, Mohammed Asselem, Omar Alsaif, Virginia, Monica Agrawal, for creating a positive and humour enriched environment. My special thanks are due to my beloved friends especially Abhi, Dhiraj, Bidha, Bhavya, Simon, Vijeth, Ariele and Rohit for sharing good and bad moments of my everyday life.

Finally I express my deepest thanks to my wife Priti and my family for always being there to listen and encourage me.

DECLARATION

I, Mr. Ashtavinayak Paradh, hereby declare that I am the author of thesis entitled, "*Pectinatus and Megasphaera*: RNA based novel detection methods." All the work done in this thesis is my own work except where stated in the text. The work done here has not been accepted in any previous application for a higher degree. All the source of information have been consulted by myself and are acknowledge by means of references.

Ashtavinayak Paradh

Edinburgh

September, 2013

LIST OF TABLES

Table: 2.1 Occurrence of beer spoilage microorganisms in different stages of the brewing process.....19

Table: 2.2 Percentage of beer spoilage incidents reports during the 1980-2002 period.....21

Table: 2.3 Characterisation of beer spoilage *Pectinatus* species.....26

Table: 2.4 Characterisation of beer spoilage *Megasphaera* species.....30

Table: 2.5 Acceptable limit of detection of beer spoilage microorganisms in the brewery.....33

Table: 2.6 Culture media for the detection of beer spoilage bacteria.....36

Table: 2.7 Summary of PCR based methods used for detection of brewery contaminants.....40

Table: 3.1 Bacterial species and strains used for the study.....52

Table: 3.2 List of primers used for detection of *Pectinatus* and *Megasphaera*.....59

Table: 3.3 List of primers used for detection *of Lactobacillus and Pediococcus*.....60

Table: 3.4 Properties of beer used for the study.....65

Table: 3.5 Sequences of oligonucleotide probes used for HPA assay and RNA slot blot analysis.....64

Table: 4.1 Multiplex PCR results for the breweries.....89

Table: 4.2 Summary of hygiene monitoring, inspection and microbial methods utilized in the breweries.....90

Table: 4. 3 Details of bacterial isolates 94

Table: 4.4 General physiological characteristics of putative isolates of *Pectinatus* and *Megasphaera*.....96

Table: 4.5 Results for antibiotic susceptibility test using mast mid-8 ring.....100

Table: 4.6 DNA probes aligned with sequence from target and closely related bacteria.....107

Table: 4.7 Labelling efficiency of DNA oligonucleotide.....112

Table: 4.8 Important parameters related to AE labelled DNA probe.....113

Table: 4.9 Half lives of AE labelled DNA probes from differential hydrolysis.....116

Table: 4.10 Effect of different pH values on chemiluminescence (S/N ratio) of AE labelled probes.....118

Table: 4.11 Hybridisation and DH conditions for AE labelled probes.....120

Table: 4.12 Effect of bacterial lysis treatment on HPA assay using PCFH-2 probe....129

Table: 4.13 Sensitivity of HPA assay in different beer (0 % and 2% ABV beer) and beer enriched medium.....132

Table: 4.14 Sensitivity of HPA assay in different beer (4 % and 6.6 % ABV beer) and beer enriched medium.....133

Table 4.15 Application of different bacterial detection methods and comprehensive detection of brewery isolates.....135

LIST OF FIGURES

Figure2.1 Global beer productions by region in financial year 2011.....7

Figure 2.2 Alcoholic drink markets in UK, 2007.....9

Figure 2.3 Schematic diagram of brewing process.....10

Figure 2.4 Dendrogram of *Saccharomyces sensu stricto* complex 14

Figure 2.5 A chemical structure of acridinium NHS ester; B- chemiluminescence reaction of acridinium NHS ester with alkaline peroxide.....44

Figure 2.6 The probable and other alternative mechanisms of chemiluminescence obtained from AE.....45

Figure 2.7 AE chemiluminescent reactions through pseudo base formation and probable alternative routes.....46

Figure 3.1 Schematic diagram of sampling points used during the project.....54

Figure 4.1 Specificity of PCR multiplex primers.....85

Figure 4.2 The sensitivity of *Pectinatus*, *Megasphaera*, *Lactobacillus* and *Pediococcus* multiplex PCR.....86

Figure 4.3 *Pectinatus* and *Megasphaera* multiplex PCR analysis of the breweries showing positive results.....88

Figure 4.4 Characteristics of brewery isolates.....97

Figure 4.5 SEM images of putative isolates *Pectinatus* and *Megasphaera*.....98

Figure 4.6 A comparison of antibiotic susceptibility profiles of putatative *M. cerevisiae* isolate (ICBD strain MC-1) with *M. cerevisiae* (DSM 20461).....99

Figure 4.7 Acetic acid and propionic acid profiles for the presumed isolates of *P. cerevisiiphilus* and *P.frisingensis*.....102

Figure 4.8 Denaturing MOPS gel electrophoresis of Bacterial RNA.....105

Figure 4.9 A 5' end amino modified AE labelled DNA probe.....108

Figure 4.10 AE labelled DNA probe with internally amino modified thymidine base.....109

Figure 4.11 Reverse Phase HPLC purification profile for end labelled PCFH-2 probe.....111

Figure 4.12 Differential hydrolysis kinetics for AE labelled probes.....115

Figure 4.13 Effect of volume of hybridisation buffer on chemiluminescence performance of internally labelled AE probes.....117

Figure 4.14 Effect of hybridisation time on performance of internally AE-labelled probes.....118

Figure 4.15 The specificity of the probes PC-2, PF-2, PH-2 and PCFH-2 probes compared to related beer spoilage microorganisms.....122

Figure 4.16 The specificity of the probes MC-2 and MPS-2 probes compared to related beer spoilage microorganisms.....123

Figure 4.17 Comparisons of sensitivity of RNA slot blot analysis and HPA assay.....125

Figure 4.18 RNA slot blot analysis and HPA assay conducted for probes PC, PF and PH probes 126

Figure 4.19 RNA slot blot analysis and HPA assay conducted for probes MC and MPS using DIG labelled probes.....127

Figure 4.20 Contribution of different beer to background noise was measured using detection step of HPA assay.....128

Figure 5.1 Relation between arousal of complaints and microbial nature of beer packaging lines.....140

Figure 5.2 Maximum parsimony phylogenetic tree for the presumed *P. cerevisiiphilus* isolates (PC-1and PC-2).....143

Figure 5.3 Maximum parsimony phylogenetic tree for the presumed *P. frisingnesis* isolates (PF-1 and PF-2).....143

Figure 5.4 Maximum parsimony phylogenetic tree for the presumed *M. cerevisiae* isolates (MC-1, MC-2 and MC-3).....144

LIST OF ABBREVIATIONS AND SYMBOLS

%	percentage
<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
µL	micro litre
µm	micro meter
ABD	Advanced beer detection medium
ABV	alcohol by volume
AE	acridinium ester
ANOVA	analysis of variance
ASBC	American Society of Brewing Chemists
ATCC	American type culture collection
ATP	adenosine tri phosphate
BBPA	British beer and pub association
BCOJ	Brewing convention of Japan
BLAST	basic local alignment search tool
bp	base pair
BRC	British retail consortium
ca.	circa (approximately)
CCV	cylindro conical vessels
CECT	The Spanish Type Culture Collection
CFU	colony forming units
CIP	cleaning in place
DH	differential hydrolysis
DIG	digoxygenin
DMSO	dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
EBC	European Brewing Convention
FAO	Food and Agriculture Organization of the United Nation
fg	femto gram
G+C	Guanine and Cytosine content
HACCP	Hazard Analysis Critical Control Points
HPA	hybridisation protection assay

IBD	Institute of Brewing and Distilling
ICBD	International Centre for Brewing and Distilling
ISO	International Organization for Standardization
L	litres
LAB	Lactic acid bacteria
LB	Luria Bertani
LNA	Locked nucleic acids
mg	milli gram
min	minutes
mM	milli meter
MOPS	3-(N-morpholino) propane sulfonic acid
MP	maximum parsimony
MRS	De Man, Rogosa, Sharpe medium
NBB	Nachweis medium für Bierschädliche Bakterien
ng	nano grams
NHS	N-hydroxy succidymiyl ester
nM	nano meter
O.D.	optical density
^{o}C	degree celsius
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PET	poly ethylene terepthalate
pmol	pico-moles
ppm	parts per million
PU	pasteurisation unit
PYF	peptone- yeast extract-fructose
QC	quality control
RLUs	relative light units
RNA	Ribonucleic acid
RNases	ribonuclease
RP-HPLC	reverse phase- high performance liquid chromatography
rpm	revolution per minute
rRNA	Ribosomal ribonucleic acid
S/N	signal to noise ratio
sec	second/s

SEM	Scanning Electron Microscopy
SMMP	selective medium for *Megasphaera* and *Pectinatus*
Taq	*Thermus aquaticus*
T_m	melting temperature
UBA	Universal beer agar
UK	United Kingdom
v/v	volume by volume
VTT	Valtion Teknillinen Tutkimuskeskus Culture Collection, Finland
w/v	weight by volume
xg	times gravity

RESEARCH PUBLICATIONS, POSTERS AND PRESENTATION

Publication

Paradh, **A**. **D**., Hill, A. E., Mitchell, W.J., Occurrence of *Pectinatus* and *Megasphaera* in UK Breweries, 2011, Journal of the Institute of Brewing, 117(4), 498-506.

Paradh, **A**. **D**., Hill, A. E., Mitchell, W. J., Detection of *Pectinatus* and *Megasphaera* using Acridinium ester labelled DNA probes, Journal of Microbiological methods, 2013 (submitted- manuscript No. MIMET-D-13-00419).

Poster Presentations

Paradh, A. D., Hill, A. E., Mitchell, W.J., Prevalence of *Pectinatus* and *Megasphaera* in UK Breweries, 33rd International congress of European Brewing Conventions, Glasgow, UK May 2011.

Paradh, A. D., Hill, A. E., Mitchell, W.J., A novel method for the detection of Gram-negative spoilage organisms in breweries, 2011, 33rd International congress of European Brewing Conventions, Glasgow, UK, May 2011.

Cowley, L., **Paradh**, **A. D**., Guerra, M. M., Hindle, L., Le Noc, M., MacLaren, H. and Fowler, R., Interaction between two species of probiotic bacteria promotes inhibition of *Clostridium difficile* growth in vitro, Society of General Microbiology symposium, Dublin, Ireland, 2012.

Presentation

Paradh, A. D., Hill, A. E., Mitchell, W.J., Detection of *Pectinatus* and *Megasphaera* using Acridinium ester labelled DNA probes, IBD, Video link day, January-2012.

TABLE OF CONTENTS

Abstract.....II
Acknowledgement.....V
Declaration.....VI
List of tables.....VII
List of figures.....IX
List of abbreviations and symbols.....XI
Publications, posters and presentation.....XIV

1. INTRODUCTION.....1
2. LITERATURE REVIEW.....3
2.1. An introduction to brewing.....5
2.2. Overview of brewing industry.....7
2.2.1. The global brewing industry.....7
2.2.2. Brewing Industry in the UK.....8
2.3. Brewing process.....10
2.3.1. Malting.....10
2.3.2. Milling.....11
2.3.3. Mashing.....11
2.3.4. Wort clarification.....12
2.3.5. Wort boiling.....12
2.3.6. Fermentation.....13
2.3.7. Conditioning and maturation.....15
2.3.8. Biological stabilisation 15
2.3.9. Packaging.....16
2.3.10. Beer dispensing.....17
2.3.11. Brewery hygiene and sanitation.....17
2.4. Beer spoilage microorganisms.....18
2.5. Gram positive bacteria.....22
2.5.1. Genus: *Lactobacillus*.....22
2.5.2. Genus: *Pediococcus*.....23
2.5.3. Other Gram positive bacteria.....24
2.6. Other Gram negative bacteria.....25
2.6.1. Genus: *Pectinatus*..... 25
2.6.1.1. Current phylogenetic status.....27

2.6.1.2. Occurrence of *Pectinatus* within the brewery environments.....27
2.6.1.3. Beer spoilage ability.....28
2.6.2. Genus: *Megasphaera*.....29
2.6.2.1. Current phylogenetic status.....30
2.6.2.2. Occurrence of *Megasphaera* within the brewery environments.....30
2.6.2.3. Beer spoilage ability..... 31
2.7. Bacterial detection methods.....31
2.7.1. Conventional methods.....32
2.7.2. Physical analysis based methods.....35
2.7.3. Biochemical analysis based methods.....36
2.7.4. Protein analysis based methods.....36
2.7.5. Immunoassay based methods.....37
2.7.6. Chromatography based methods.....37
2.7.7. Nucleic acid analysis based methods.....38
2.7.7.1. Molecular probes..... 38
2.7.7.2. Automated ribotyping 38
2.7.7.3. Polymerase chain reaction based methods 39
2.8. Hybridisation Protection Assay.....42
2.8.1. Acridinium esters 43
2.8.2. Mechanism of chemiluminescence of acridinium ester.....43
2.8.3. Working principle of Hybridisation Protection assay.....47
2.8.4. Hybridisation Protection Assay Formats.....47
2.8.5. Advantages and Disadvantages of HPA assay.....48
2.9. General application of HPA assay.....48

3. MATERIALS AND METHODS
3.1. Bacterial strains and culture conditions.....51
3.2. Brewery samples.....53
3.2.1. Swab samples.....55
3.2.2. Beer and rinse samples.....55
3.2.3. Yeast slurry samples.....55
3.3. Sample enrichment and cell harvesting.....55
3.4. Genomic DNA extraction and quantification.....56
3.4.1. DNA extraction.....56
3.4.2. DNA quantification.....57

3.5. Multiplex PCR.....57
3.5.1. *Pectinatus* multiplex.....57
3.5.2. *Megasphaera* multiplex.....57
3.5.3. *Lactobacillu*s multiplex.....58
3.5.4. *Pediococcus* multiplex 58
3.5.5. PCR conditions and Gel electrophoreses.....61
3.5.6. Determination of specificity of primers.....62
3.5.7. Determination of sensitivity of primers.....62
3.6. Isolation of *Pectinatus* and *Megasphaera* isolates.....62
3.7. Characterisations of *Pectinatus* and *Megasphaer*a isolates.....63
3.7.1. General Characterisation.....63
3.7.2. Scanning electron microscopy.....63
3.8. Biochemical characterisations.....64
3.8.1. Carbohydrate utilisation profiling.....64
3.8.2. Determination of bacterial growth at different pH.....64
3.8.3. Determination of bacterial growth at different temperatures.....64
3.8.4. Antibiotic susceptibility.....65
3.8.5. Determination of beer spoilage ability..... 65
3.8.5.1. Determination of beer bitterness.....66
3.8.5.2. Determination of concentration of acetic acid and propionic acid.....66
3.9. Genetic characterisation.....66
3.9.1. Amplification of 16S ribosomal gene.....66
3.9.2. Partial 16S ribosomal gene sequencing.....67
3.9.3. Analysis of 16S ribosomal gene sequence.....67
3.10. Hybridisation Protection Assay.....68
3.10.1. Preparation of working cultures 68
3.10.2. RNA stabilisation.....68
3.10.3. Total cellular RNA isolation.....68
3.10.4. Total cellular RNA quantification..... 69
3.10.5. Determination of quality of RNA.....69
3.11. Selection and Modification of DNA probes.....69

3.11.1. 5’ end amine modification of DNA probes.....72
3.11.2. Internal amino modification DNA probes.....72
3.11.3. Ethanol precipitation of the probe.....72

3.12. Labelling and Purification of DNA probes.....72
3.12.1. DNA oligonucleotide labelling.....73
3.12.2. Reverse phase HPLC purification.....73
3.13. Determination of specific activity of AE probes.....74
3.13.1. Measurement of chemiluminescence.....74
3.13.2. Determination of concentration of AE- labelled probes.....74
3.14. Determination of DH kinetics of AE probes.....75
3.15. Optimisation of HPA assay.....76
3.16. Optimised HPA used in the study.....76
3.16.1. Hybridisation.....76
3.16.2. Differential hydrolysis.....76
3.16.3. Detection.....76
3.17. Additional HPA protocol.....77
3.18. Slot blot analysis of RNA using Dig labelled probes......77
3.18.1. Slot blotting of RNA samples.....77
3.18.2. Prehybridisation and hybridisation.....77
3.18.3. Stringency washing.....77
3.18.4. Blocking.....78
3.18.5. Detection.....78
3.19. Determination of Specificity of AE probes..... 78
3.20. Determination of Sensitivity of AE probes.....78
3.21. Bacterial cell lysis protocols.....79
3.21.1. Enzymatic lysis.....79
3.21.2. Detergent lysis 79
3.21.3. Heat lysis.....80
3.22. Applications HPA assay in Brewing laboratory.....80
3.22.1. Determination of background noise from different beer.....80
3.22.2. Sensitivity of HPA assay for beer and beer enriched media.....80
3.23. Analysis of real brewery samples.....81
3.24. Statistical analysis.....81

4. RESULTS.....82
Results Part I Occurrence of *Pectinatus* and *Megasphaera* in UK breweries.....82
4.1. Optimisation of multiplex PCR methods.....83
4.2. Specificity of multiplex PCR.....84

4.3. Sensitivity of multiplex PCR.....84
4.4. Multiplex PCR results.....86
4.5. Hygiene and sanitation the breweries.....92

Results Part II Characterisation of putative isolates of Pectinatus and *Megasphaera* 93
4.6. Isolation of *Pectinatus* and *Megasphaera* isolates 94
4.7. General physiological characterisation.....94
4.8. Morphological characterisation.....94
4.9. Scanning electron micrographs.....98
4.10. Antibiotic susceptibility tests.....99
4.11. Determination of beer spoilage ability.....101
4.12. Genetic characterisation.....103

Results Part III Hybridisation Protection assay.....105
4.13. RNA isolation and stabilisation..... 105
4.14. Probe selection and modification.....106
4.14.1. 5'amino modified probes..... 108
4.14.2. Internal modified probes.....109
4.15. Labelling and purification of DNA probes.....109
4.16. Specific activity of AE labelled DNA probes..... 112
4.17. Determination of DH of AE labelled DNA probes.....113
4.18. Optimisation of HPA assay.....116
4.18.1. Effect of volume of hybridisation buffer.....116
4.18.2. Effect of time of hybridisation.....117
4.18.3. Effect of pH and time of differential hydrolysis DH kinetics.....118
4.19. Specificity of AE labelled DNA probes..... 120
4.20. Sensitivity of AE labelled DNA probes.....123
4.21. Application in the brewing laboratory.....127
4.21.1. Detection of background noise.....127
4.21.2. Comparative results for cell lysis protocol.....128
4.21.3. Bacterial CFU detection limit using HPA assay.....130
4.21.4. Sensitivity of HPA assay in different beer and beer enriched media.....130
4.22. Detection of brewery isolates of *Pectinatus* and *Megasphaera* 132

5. DISCUSSION

5.1. Discussion Part I Occurrence of *Pectinatus* and *Megasphaera* in the major UK breweries.....138

5.2. Discussion Part II Characterisation of putative isolates of *Pectinatus* and *Megasphaera* 142

5.3. Discussion Part III Hybridisation Protection assay 145

5.3.1. Introduction.....145

5.3.2. Probe selection and modification…….....145

5.3.3. Comparison of Developed HPA assay and RNA slot blot analysis.....146

5.3.4. Bacterial CFU detection limit of HPA assay.....147

5.3.5. Performance of HPA with beer enrichment media ……..…..…147

5.3.6. Detection of brewery isolates of *Pectinatus* and *Megasphaera*.....148

5.4. Conclusion and further study.....150

6. APPENDICES.....152

Appendix- I List sampling points and multiplex PCR results.....153

Appendix -II Sugar utilisation profiles for putative isolates of *Pectinatus* species.....159

Appendix- III Partial ribosomal gene sequences of the isolates.....162

III A -Partial 16S ribosomal gene sequence (ICBD strain PC-1) aligned.....162

III B- Partial 16S ribosomal gene sequence (ICBD strain PC-2) aligned.....163

III C- Partial 16S ribosomal gene sequence (ICBD strain PF-1) aligned.....164

III D- Partial 16S ribosomal gene sequence (ICBD strain PF-2) aligned.....165

III E- Partial 16S ribosomal gene sequence (ICBD strain PF-3) aligned.....166

III F- Partial 16S ribosomal gene sequence (ICBD strain MC-1) aligned.....167

III G- Partial 16S ribosomal gene sequence (ICBD strain MC-2) aligned.....168

III H- Partial 16S ribosomal gene sequence (ICBD strain MC-3) aligned.....169

7. REFERENCES.....170

8. PUBLISHED PAPER.....199

CHAPTER: 1 INTRODUCTION

CHAPTER 1 INTRODUCTION

Beer is considered as a microbiologically stable beverage. Several antimicrobial factors such as low pH (3.8-4.7), presence of ethanol (0.5 % -10 % (w/v)), presence of Hop bitterness compounds (iso-α acids), low oxygen concentration (less than 0.1- 0.3 ppm), relatively high CO_2 (0.5 % (w/v)) (Jespersen & Jakobsen, 1996) and low levels of nutrients make propagation of contaminants difficult in beer (Sakamoto and Konings, 2003). In addition, technological and processing hurdles such as wort boiling, pasteurisation and sterile filtration ensure that most food borne pathogens do not grow or survive in beer (Dowhanick, 1994, Menz *et al*., 2009).

There is a narrow range of non pathogenic beer spoilage bacteria which can still survive, grow and spoil beer (Strogårds, 2006). Bacteria belonging to genera *Lactobacillus*, *Pediococcus*, *Pectinatus* and *Megasphaera* represent major group of beer spoilage microorganisms (Ijima, 2008). Very few cases of beer spoilage have been reported in recent years due to high standards of hygiene and technological improvements within the brewing industry. However, due to the extent of consumer awareness about food and beverage safety and maintaining corporate brand image, beer spoilage microorganisms are of serious concern to breweries worldwide.

Conventional microbiological detection methods are based on selective staining followed by microscopic analysis and simple biochemical and physiological analysis methods such as nutrient assimilation, selective growth on specific agar and sensory evaluation (Hill, 2009; Juvonen, 2009). Conventional microbial detection requires long incubation times and often leads to incomplete and misleading results. As a consequence, more sensitive and rapid detection methods have gained importance in recent years (Hill, 2009).

Several rapid detection methods have been developed in recent years (Hill, 2009; Suzuki, 2012). PCR has emerged as powerful tool for detection of microbial contamination in food and beverage industry (Russell and Stewart, 2003). Despite advantages of rapid detection methods their application in brewing industry remains limited. High cost to benefit ratio, high instrumentation cost and requirement of skilled personnel are major drawbacks of these rapid methods.

The ATP bioluminescence method is routinely used in most of the breweries (Ehrenfeld *et al*., 1996; Franken *et al*., 2000). The luminometer is the basic instrument required for the ATP bioluminescence method and is therefore readily available in both medium size and large breweries. An application of a luminometer based method for detection of brewery contaminants using a highly sensitive chemiluminescence based method assay could be an easy and effective approach. Hybridisation Protection assay (HPA) is nucleic acid hybridisation based method, which involves hybridisation of chemiluminescent acridinium ester (AE) labelled DNA probes to target nucleic acid, followed by a differential hydrolysis step which involves alkaline hydrolysis of free and unhybridised AE probes while the hydrolysis of hybrid AE probe is prevented. The final step involves measurement of the chemiluminescence signal, which is directly associated with the hybridized probes. A simple in-solution protocol, high sensitivity, specificity and versatility are the main advantages of the HPA assay (Nelson *et al*., 1998). The data available for HPA assay are quantitative, reliable and reproducible hence the results are easy to analyse. The HPA assay can be applied to the detection of DNA and RNA molecules from diverse sources; hence these assay formats are versatile and show compatibility for use in clinical, pharmaceutical, food and beverage industries.

The study primarily aimed at investigation of occurrence of Gram negative, anaerobic beer spoilage microorganisms belonging to the genera *Pectinatus* and *Megasphaera* in the major UK breweries. The first part of the study aimed to optimise of multiplex PCR and to use the optimised methodology for detection of beer spoilage *Pectinatus*, *Megasphaera*, *Lactobacillus* and *Pediococcus* species. The putative isolates of *Pectinatus* and *Megasphaera* were intended to be characterised initially using routine microbiological methods and then identified using partial 16S ribosomal gene sequencing.

The main aim of this study was to then develop a luminometer based, chemiluminescence method for rapid detection of beer spoilage microorganisms belonging to the genera *Pectinatus* and *Megasphaera*. Acridinium ester labelled DNA probes were used for genus and species specific detection of these microorganisms. The project also focussed on evaluation of specificity and sensitivity of the developed acridinium ester labelled DNA probes. Finally the study aimed at development of routine protocols for different brewery environment samples and evaluated compatibility of routinely used beer enrichment media with the developed HPA assay.

CHAPTER: 2 LITERATURE REVIEW

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction to brewing

Beer is an alcoholic beverage produced by enzymatic conversion of starch from cereals into fermentable sugars followed by fermentation of these sugars to alcohol and other by-products by the action of yeast. Hops are added mainly for bittering and flavouring. Brewing is one of the basic and oldest applications of biotechnology with beer being one of the oldest alcoholic beverages (Briggs *et al.*, 2006; Campbell, 2003). Although the origin of brewing is thought to be almost 10,000 years ago (Axcell, 2007), the archaeological evidence of the existence of brewing can be traced back to 6000-7000 years ago (Katz and Maytag, 1991; Anderson, 2003). In 1800 BC brewing was limited to domestic activities of the agrarian population in Sumerian civilisation using breads made with the *Triticum dicoccum* grain variety which were soaked in water and spontaneous fermentation was supposedly carried out due to activity of wild yeasts (Esslinger and Narziss, 2009). Later, in the Babylonian era, barley and emmer were used for brewing a variety of beers. Egyptians gradually modified the brewing process by germinating grains specifically for brewing, thus eliminating the use of soaked bread. They also had legal regulations for beer brewing practices. These ancient communities had knowledge of yeast being the preferred agent for brewing but biochemical and physiological processes are assumed to have been unknown (Lodolo *et al*., 2008).

From the 14th century hops were used as bittering and flavouring additives in beer (Esslinger and Narziss, 2009). During the 15th and 17th century hops were introduced in Britain and North America, respectively. Later in the 17th century hopped beer became dominant all over Europe (Anderson, 2003).

In 1516, the Reinheitsgebot (Purity Law), the law to regulate production of beer was introduced which can be considered as the first law related to food and beverage production. This purity law defines the production of beer from malted barley, hops, water and yeast. Yeast may be introduced only as an organism for fermentation and addition of any other additives is strictly prohibited. After 1516 the purity law regulated the production of beer in Germany, Sweden and Greece (Esslinger and Narziss, 2009).

Until the 16th century, beer fermentation used to be spontaneous and carried out at high temperature using top fermenting yeast. In 1680 with the development of the microscope, Antoine van Leeuwenhoek for the first time described the morphology of brewing yeast and also stated the phenomenon of beer spoilage (Lodolo *et al*., 2008).

Extensive research in brewing and malting took momentum from the 17th century (Esslinger and Narziss, 2009). Industrial scale production of beer started towards the end of the 19th century with the introduction of pure yeast strains by Emil Christian Hansen (Lodolo *et al*., 2009; Polaina, 2002). He also distinguished between top and bottom fermenting yeast based on their morphology and fermentation characteristics (Rank *et al.,* 1988).

Beer production today is a result of technological changes in malting, brew house technology, fermentation, filling and packaging innovations. In addition to enhanced quality and reliability in beer production, breweries in recent years are not only focused on improvement of cost effective measures in production but also in development of stable, secure, safe and environmentally friendly procedures (Andrews *et al.,* 2011).

Due to the extent of beer production in large batches, consumer awareness of product quality and increasing competition within industry, biological and non biological stability has become a crucial issue. Beer is considered a microbiologically safe beverage due to its antimicrobial properties and effective sanitation practices utilised in breweries (Strögards *et al*., 2006). A limited number of spoilage microorganisms can still spoil the beer. Incidents of beer spoilage can hamper the beer brand, cause loss of consumer loyalty and can also cause serious economic losses. Disposal of spoiled beer can also be an environmental issue. In addition, due to an increase in production of low alcohol, alcohol free and unpasteurised beer, microbiological quality has become even more important (Asano *et al*., 2008). There has been remarkable improvement in the development of rapid detection methods for spoilage organisms based on various new techniques within the last decade (Hill, 2009). Most of the rapid detection methods are expensive and initial investment is quite high; these methods often require technical expertise and constant technical improvements require revision and improvement of protocols. Due to all these factors microbiological quality assurance (QA) in breweries is still carried out using conventional methods and application of rapid detection methods is limited. There has always been scope for development of easy, simple to operate, highly sensitive and cost effective methods for microbiological detection.

2.2 Overview of the Brewing Industry

2.2.1 The Global brewing industry

Beer is the third most favoured drink across the globe after water and tea and the most favourite alcoholic beverage. There have been recent changes in the global beer market due to emergence of developing markets such as China, Brazil and India and merging and acquisition of small regional breweries into a few multinational brewing companies. In 2010 the four largest brewing companies; Anheuser-Busch InBev (AB- InBev), SABMiller, Heineken and Carlsberg were responsible for more than 50 % of beer production across the globe and 70 % of the revenue of the brewing industry

Global beer production in the year 2011 amounted to 1927.1 million hectolitres, showing a growth of 3.7 % compared to beer production in 2010. The global beer production in financial year 2011 is shown in Figure 2.1. In the same financial year beer production in developed markets such as Europe and North America showed a decrease by 0.2 % and 1.5% respectively. On the other hand new emerging markets such as Asia and Latin America showed high increases in production by 8.6 and 3.1 % respectively compared to 2010 (Kirin Institute of Food and Lifestyle Report, 2012).

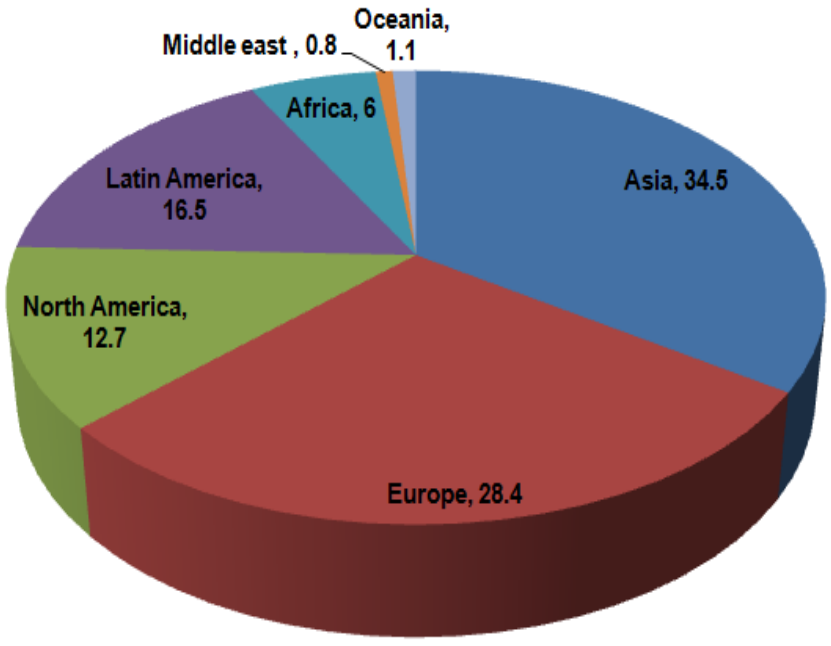


Figure 2.1 Global beer production by region in financial year 2011 (Source - Kirin Institute of Food and Lifestyle Report, 2012)

In 2007, barley production was estimated to be 148 million tonnes, accounting for 26 million tonnes of malting barley. Out of 21 million tonnes of barley malt produced, 19.5million tonnes was used for beer production, 0.9 and 0.6 million tonnes were used for whisky production and food purpose respectively (FAO report, 2009).

In 2004, the major malting industries based on production were Groupe Soufflet (France), Groupe Malt Europ (France), Cargill Incorporation (USA), United Malt Holding (USA/ Canada), Lesaffre-ADM (Archer Daniels Midland Company)/IMC (International Malting Company) (USA) accounting for 33.9 % of overall global market production (Esslinger and Narziss, 2009) . The production share of these large malting companies is comparatively low due to the fact that various multinational brewing companies have their own malting facilities and also these breweries purchase malt from other regional suppliers to reduce transportation costs (FAO report, 2009).

In 2011, global hops production was documented to be 100, 603.9 metric tonnes and the total alpha acid equivalent production of 10,348 metric tonnes. In the same year Germany, USA and China were the major hop producers accounting for 36.1, 36.1 and 8.7 % of global alpha acid equivalent production. (The Barth report, 2011)

Unlike the malting industry the hops industry is dominated by just four major companies; Barth- Haas group, Hopsteiner group, Yakima chief and HVG (Hopfen Verwertungs Genossenschaft) accounting for 85 % of total hops production in 2006 (Esslinger and Narziss, 2009).

2.2.2 The UK brewing industry

Until the 1980s beer production within the UK was mainly concentrated in national breweries and regional breweries producing predominantly real ales and other dark beers. Since the 1990s with the beginning of consolidation of small and regional breweries into multinational brewing companies the scenario of the UK beer industry has changed. The UK beer market over the last decade has shown a gradual decrease in production due to competition faced by other alcoholic drinks such as wine, cider and other flavoured alcoholic beverages, increased taxation and a ban on public smoking (Key Notes, 2008). The alcoholic drink market in 2007 is shown in Figure 2.2. In 2011 beer production amounted to 45.69 million hectolitres with 1.5 % positive growths compared to the beer production in 2010 (Kirin Institute of Food and Lifestyle Report, 2012).

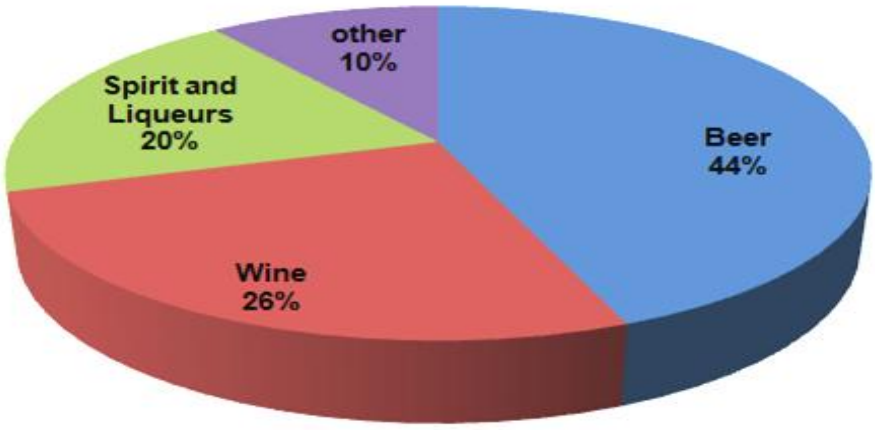


Figure 2.2 Alcoholic drink markets in UK, 2007 (based on retail selling prices). (Source- Keynote, 2008)

Despite the decrease in annual beer production, in 2011 the UK had over 900 operational breweries, including major breweries, regional breweries, microbreweries and brewpubs, and the UK brewing and pub industry was estimated to be worth £ 19.4 billion providing employment to an estimated 984,000 personnel (BBPA, 2011).

In terms of volume, lager accounted for 56.8 % and dark beers accounted for 43. 2 % of the total UK beer market in 2012. Lager accounted for 71.5 % (value - retail selling price) of the total beer market in the UK and is predicted to reach 88 % by 2015 due to the recent increase in off trade sale of beer (Key note, 2012)

2.3 The brewing process

The main ingredients used in production of beer are malted barley, hops, water and yeast. The steps employed in the production vary according to variety of beer style and type based on raw materials and microbial agents during fermentation. The general steps involved in a typical brewing process are described in Figure 2.3.

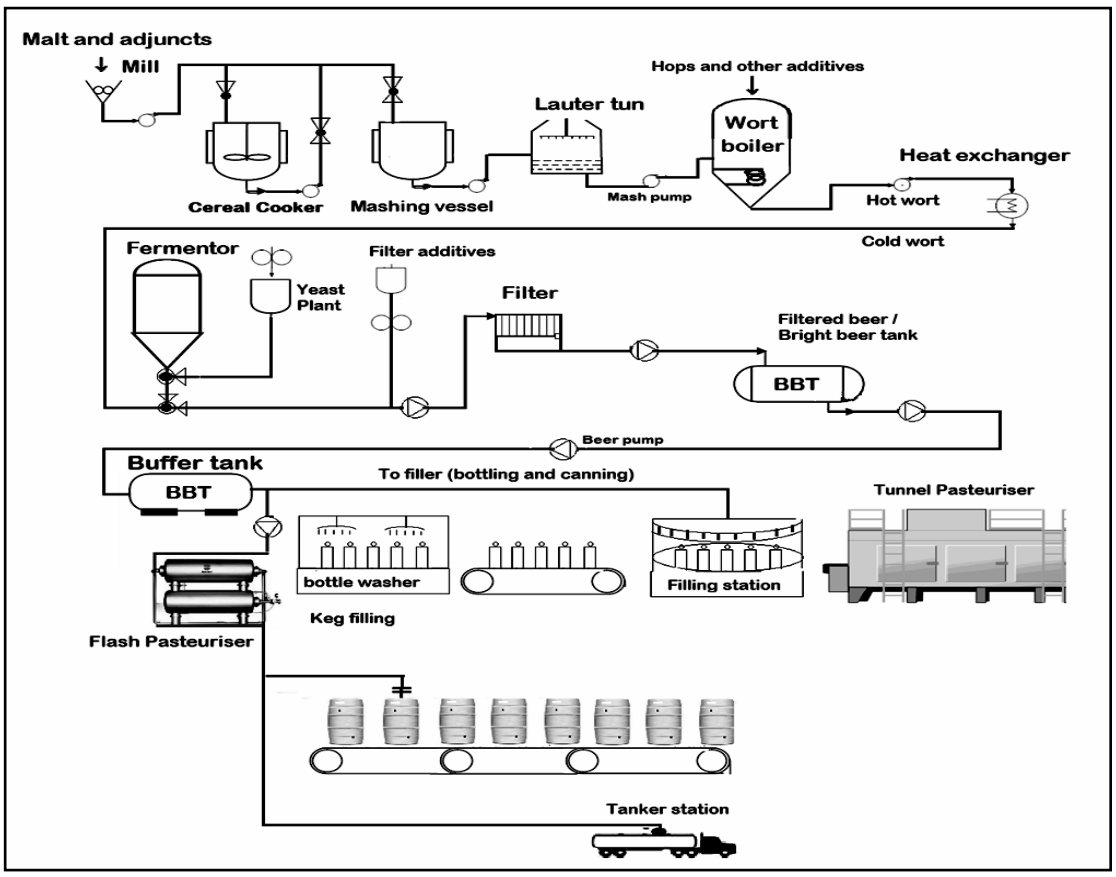


Figure: 2.3 Schematic diagram of the brewing process

2.3.1 Malting

Malt production is a separate industry and most of the malt produced is used in brewing and distilling, but malt can also be used in bakery and other food productions. The malting process converts raw cereals such as barley (typically used in brewing and distilling), wheat, oats, rice, sorghum and rye into a chemically and physiologically altered processed grain with a high enzyme content called malt (Palmer, 2003). Barley is generally preferred over other cereals due to control of germination and it is also preferred in terms of taste.

Malting involves steeping of grains in cold water followed by initiation of germination in a natural way. During germination the grain embryo produces gibberellins, a plant hormone, which activates the aleurone layer of the grain to produce various enzymes essential for degradation of grain starchy endosperm. The germination process is typically stopped by kilning (heat treatment). Kilning is important as it gives malt a typical colour and flavour. Malted barley basically provides starch and protein degrading enzymes during the mashing process but speciality malts are used to enhance colour, aroma and flavour of beer depending on desired beer style.

2.3.2 Milling

Milling is a critical step for increasing yield extract during mashing (Eaton, 2003). Malt and other whole grain brewing adjuncts are milled to break down malt and grain to allow penetration of carbohydrate degrading enzymes to degrade starch into fermentable sugars. Milling is basically carried out using roller, hammer or wet mill depending upon the separation step to be utilised (Kuzne, 1996). The roller mill allows grinding of malt into typical grist ratio of course, fine and flour which later is desirable in the lautering process to separate wort from other malt and adjunct solids (Eaton, 2003). Wet milling reduces the damage to the husk and also efficiently separates endosperm from husk making it available for mashing process (Lewis and Young, 2002).

2.3.3 Mashing

The process of mashing involves mixing of malt grist and other sources of fermentable sugar with hot liquor (water) to allow breakdown of starch in to fermentable sugars. The temperature of mashing typically lies between 62-65 ºC to ensure optimum activity of starch degrading enzymes but can be varied according to the process. Hot water causes swelling of starch kernels and breakdown of crystalline starch granules into gelatinised liquid, the process in called gelatinisation or liquefiction (Esslinger and Narziss, 2009). Mashing is generally carried out for 1 hour and breakdown of starch to fermentable sugars is checked using an iodine test, α-glucans (dextrins) reacts with iodine to give blue colour indicating incomplete breakdown of starch and negative iodine test indicates desired attenuation limit is achieved (Esslinger and Narziss, 2009). The malt enzymes break up the starch molecules into fermentable sugars such as glucose, maltose and maltotriose, and these sugars dissolve in the liquor surrounding the mash to form a sugary solution generally designated as wort. Wort composition can vary due to changes in raw materials and other processing factors (Gunkel *et al.*, 2002; van Nierop *et al.*, 2006).

There are various mashing methods which have been developed according to mashing equipment, materials available and beer styles. Infusion mashing, decoction mashing and double mashing are important mashing processes typically used in the UK, Europe and America respectively (Eaton, 2003).

2.3.4 Wort clarification

Before wort boiling, the wort is separated from residual solids from the mashing process. Wort separation can be carried out in the same vessel used for mashing (mash tun) or another vessel called a lauter tun and can also be separated using mash filters. A lauter tun is wide diameter vessel with a false bottom. Residual grain solids form a shallow bed at the bottom of vessel which allows separation of wort from residual grain solids. In lautering the mash is sparged with hot liquor to maximise the recovery of fermentable sugars (Eaton, 2003).

In mash filters pressure is applied to separate wort from residual solids. The use of mash filters has been reported in breweries for many years (McElevey, 1974). Recent innovative mash filters (Meura 2001) provide effective membrane filters, fast and effective process control which has maximised the extract recovery by reducing moisture content of spent grains (Melis, 2001). In recent years about 25 % of global beer production is produced using mash filters (Andrews, 2004).

2.3.5 Wort boiling

Wort boiling involves boiling of wort with hops to impart bitterness, flavour and aroma to beer. Wort boiling also ensures sterilisation of wort from microbiological contaminants (Andrews and Axcell, 2003). Wort boiling causes thermal destruction of vegetative cells and their spores survived during mashing regimes (Vriesekoop *et al*., 2013). Wort boiling also converts hop acids from hops added early during boiling (bittering hops) into iso alpha acids (isomerised) which give beer a typical bitterness and hops added late during boiling provide aroma and flavour due to hop oils. Hop bitterness compounds are also known to inhibit the growth of Gram positive bacteria. Hop derived compounds induce leakage in cell membrane of Gram positive bacteria interrupting various metabolic activities of cell. Wort boiling is also important to precipitate haze precursors and remove undesirable volatile compounds such as precursors of dimethyl sulphide (DMS). Wort boiling also concentrates the wort due to evaporation of water, increasing the specific gravity of wort (Andrews and Axcell,

2003). Wort boiling is an energy intensive process which accounts for 20-40 % of thermal energy utilised in the brewery (Andrews, 2011).

After wort boiling, coagulated material formed during boiling (hot break) and hop residues are separated using several methods such as hop strainer, centrifugation, filtration, sedimentation and whirl pooling. The clear wort obtained is cooled down to pitching temperature (8-17 ºC) through a heat exchanger depending on the fermentation requirement.

2.3.6 Fermentation

After wort clarification the wort is oxygenated by injecting pure air and brewing yeast is pitched to start the fermentation process. Fermentation is an anaerobic process where brewing yeast utilises fermentable sugars from wort to form ethanol and CO_2 as the main products, various other metabolic by-products such as higher alcohols, esters and aldehydes are also produced which give characteristic flavour and aroma to the beer. Yeast viability and vitality are important factors for desired fermentation profile (Pratt *et al*., 2003). The age of the yeast culture also plays a predominant role in the fermentation performance (Powell *et al*., 2003).

The majority of yeast strains used in food and beverage fermentations belong to the *Saccharomyces sensu stricto complex* (Vaughan *et al*., 1998; Sicard and Legras, 2011) and brewer's yeast strains are generally termed as *S. cerevisiae* as taxonomic terms have always been complex and dynamic (Quinn, 2008; Lodolo, 2008). There are two main types of yeast strain used in the brewing industry; top fermenting (ale and weiss yeast) (Lodolo, 2008; Jentsch, 2007) and bottom fermenting yeast (lager yeast) (Rainier *et al*., 2006) based on their flocculation character. Besides flocculation, ale, weiss and lager yeast strains can be distinguished based on several characters such as fermentation flavour profile, cell and colony morphology, optimum growth temperatures and utilisation of specific sugars. Brewing yeast strains have also been genetically characterised and differentiated using karyotyping (Casey, 1996) and DNA fingerprinting (Wightman *et al.,* 1996).

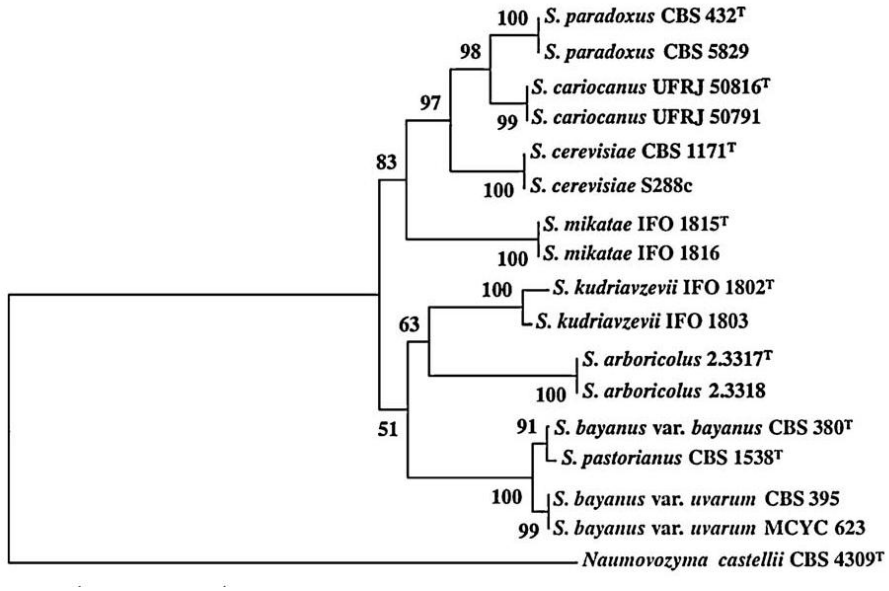


Figure 2.4 Dendrogram of *Saccharomyces sensu stricto complex* (adapted from Sicard and Legras, 2011)

Ale fermentation is typically carried out using a top fermenting strain of brewing yeast (*S. cerevisiae*). The fermentation is carried out at relatively high temperature (18-24 ºC) and at the end of the fermentation process the yeast is cropped from the top of fermenters (Lodolo, 2008). Traditionally in the UK ale fermentation was carried out within various fermentation systems such as Burton Union systems, Yorkshire Squares and Open Squares but in recent years cylindroconical vessels have come to dominate (Boulton and Quain, 2006). Ales are strong, robust in flavours and aroma due to the variety of malts and hops used in the ale production.

Lager fermentation is carried out at relatively lower temperature (8-14 ºC) using a bottom fermenting brewing yeast strain (*S. pastorianus* formerly known as *S. uvarum var carlsbergensis*) (Quain, 2008). Lager yeast strains form cell clumps (flocs) which settle down at the bottom of fermentation vessels hence cylindro conical vessels (CCV) are preferred for lager fermentation (Lodolo, 2008). The sedimentation of yeast clumps at the bottom of fermenters is termed "flocculation" (Verstrepen, 2003); it is a strain dependent character of brewer's yeast which is genetically controlled by the FLO gene cluster (Teunissen *et al.,* 1995). Flocculation is also affected by various physical and physiological factors such as temperature, agitation, pH, and osmolality of the medium and carbohydrate concentrations (Verstrepen, 2003; Strauss, 2005). As the lager fermentation is carried out at low temperature this inhibits the production of by-products such as esters responsible for fruity flavours which gives lager a crispier, light and mellow taste compared to ales.

Pitching yeast is common source of contaminants which can transfer spoilage microorganisms from fermentation to fermentation (Hill, 2009). Practice of acid washing can be effective at eliminating bacterial contaminants but still wild yeast remains unaffected.

2.3.7 Conditioning and maturation

The green beer (fermented wort before maturation) is matured to obtain desired final flavour and aroma, colloidal stability and clarification (Quain and Smith, 2009). Maturation also helps to reduce off flavour compounds such as diacetyl, SO_2 and dimethyl sulphide (DMS) aids yeast sedimentation and carbonation of beer and enhances colloidal stability.

Maturation can be broadly classified as short term warm maturation (Ruhr) and long term cold maturation (Lagering) (Lodolo, 2008). The duration of warm maturation and cold maturation ideally should be around 2-3 days and 10-90 days respectively but can vary according to beer brands (Quain and Smith, 2009). Warm maturation is carried out to reduce off flavour compounds such as diacetyl and 2, 3 pentadioane. At higher temperature yeast reduces diacetyl to acetoin and butanediol which are comparatively less flavour active than diacetyl (Quain and Smith, 2009; Lodolo, 2008).

Cold maturation is generally carried out at a temperature of 0 to -2 ºC. Cold maturation involves slow and controlled secondary fermentation primarily due to low temperature, low residual concentration of sugars and low yeast count in green beer. Fermented wort (green beer) obtained after primary fermentation is matured at low temperature to enhance flavour and clarity of a beer. Beer is stored at low temperature in this process and chill haze formed by polypeptides and polyphenols is removed from the beer.

In addition some beers are cask conditioned or bottle conditioned where secondary fermentation is carried out in beer filled casks and bottles respectively.

2.3.8 Biological stabilisation

Matured beer is filtered to remove various solid and colloidal particles such as yeast cells, protein-tannin complex (haze) and hop resins. Filtration also imparts biological, chemical and physical stability to beer (Esslinger and Narziss, 2009). Pre-filtration is mainly carried using out using filters such as plate and frame filters, pressure leaf filters or candle filters, or centrifugation. For pre-filtration using filters, diatomaceous earth or perlite is used as the filtering agent (Quain, 2008).

Final clarification is mainly carried out using filter sheets made up of cellulose and diatomaceous earth. Sterile filtration is carried out using cellulose filter sheets of specific pore size (0.45 µm) to remove microbiological contamination (Quain, 2008).

During the last decade, cross flow filtration systems using semi permeable membranes have been developed (Andrews, 2011). These systems have been reported to improve flavour stability of beer and are also found to be environmentally safe to dispose (Breons *et al*., 2008; Gaub *et al.*, 2008).

Before packaging, beer may be pasteurised using flash pasteurisation or tunnel pasteurisation. Flash pasteurisation is typically carried out in bulk and tunnel pasteurisation is typically carried out post packaging in small volumes such as cans and bottles. Beer can also be filtered using 0.45 µm size filters to ensure microbiological stability.

2.3.9 Packaging

Beer packaging can be in large volume such as casks, kegs and bulk tankers. Stainless steel casks and kegs are typically used in brewing but casks and kegs of aluminium and wood are also used (Eaton, 2003). Small packaging includes cans and bottles. For beer bottling, glass has been intensively used but recently use of PET (polyethylene terepthalate) bottles has increased due to their light weight and safe handling compared to glass (Eaton, 2003). Cans used in beer filling are mainly made from aluminium and tin plate. Worldwide 65 % of beer produced is packaged in glass bottles, canned beer accounts for 20 % of the beer market; draught beer in kegs and barrels accounts for 12 % and 3% of beer produced is marketed in PET bottles (Esslinger and Narziss, 2009).

In aseptic packaging operation in which is beer is flash pasteurised or sterile filtered before bottling or canning could be susceptible to contamination after biological stabilisation (Simpson, B., personal communication). Beer bottling and canning lines can be susceptible to beer spoilage through environmental contaminants, airborne contaminants and biofilm formation which can act as a reservoir for beer spoilage microorganisms (Timke, 2005). Microbial contamination in packaging lines can be effectively controlled routine cleaning and sanitisation of brewery conveyors and equipments. Periodic cleaning and rinsing of fillers with chlorine dioxide treated water could help in controlling microbial contamination (Dirksen, 2005).

2.3.10 Beer dispensing

Draught beer accounts for 12 % of total beer produced in the world but the volume of draught marketed in kegs and casks is considerably higher in European markets (Boulton and Quain, 2006). Draught beer accounted for 60% of total beer marketed in the UK in 2010 (BBPA statistical data, 2010). Draught beer is the most eco-friendly and cost effective way of beer sale as kegs and casks are returnable and refillable (BBPA statistical data, 2010).

2.3.11 Brewery Hygiene and Sanitation

Sanitation is important in breweries as wort or beer posses risk of getting contaminated with bacteria or wild yeast leading to off-flavours and off-aromas (Dirksen, 2005). Controlling growth of microorganisms is main objective of implementing hygiene and sanitation in breweries. The term hygiene refers to degree of cleanliness that eliminates all vegetative cells. In breweries cleaning of in fermentation tanks and bright beer tanks and other storage vessels is carried out using alkali or acid based detergents. Hot or cold caustic (0.5-2 %) CIP (cleaning in place) containing chelating and wetting agents are generally used in the breweries to clean the vessels and pipes, sometimes chlorinated caustic CIP is used to deal with hard to clean surfaces such as heat exchangers (Colosia, 2004). Acid CIP (predominantly phosphoric acid and nitric acid) is generally used for cleaning and removal of beer stone mineral deposits and scales made from metals salts (Praeckel, 2009).

Sanitising agents are used to kill the microorganisms and inhibit their growth to acceptable level. There are different types of sanitisers and their commercial formulates are available for area specific applications in food and beverage industry. Peracetic acid and hydrogen peroxide are common non rinse based sanitiser used for vessel and environment sanitisation (Holah, 1992). Chlorine dioxide has been widely used as a sanitiser in water disinfection, rinse water for packaging lines and pasteurisers (Dirksen, 2003) Quaternary ammonium salts, iodophores and sodium hypochlorite are other widely used environmental sanitisers (Praeckel, 2009). The cleaning techniques can be variable depending on capacity and automation of breweries, common techniques involve manual cleaning, high pressure cleaning, foam cleaning and automated/ semi automated CIP.

2.4 Beer spoilage microorganisms

Several antimicrobial factors present in a beer make it a microbiologically stable beverage. Beer is acidic (pH 3.8-4.7). Ethanol content of beer can vary from 0.5 % - 10 % (w/w). Ethanol causes cellular membrane damage, it also causes denaturation of proteins, interfering with metabolism and causing cell lysis of bacteria (Larson and Morton, 1991; McDonnell and Russell, 1999). Hop bitterness compounds (iso-α acids) are present at approximately 17-55 ppm of iso-α acids and can cross the cytoplasmic membrane of bacteria in their undissociated form. These compounds act as a protonophores dissipating the transmembrane pH gradient, which inhibits growth of hop sensitive microorganisms (Simpson *et al*., 1992; Simpson *et al*., 1993). The presence of low oxygen concentration (less than 0.1- 0.3 ppm) and relatively high CO_2 (0.5 % (w/v)) (Jespersen & Jakobsen, 1996) makes beer almost anaerobic. Beer also has very low levels of nutrients as most of the fermentable sugars are utilised by brewing yeast during fermentation, making propagation of contaminants difficult in beer (Sakamoto and Konings, 2003).

In addition, technological and processing hurdles such as wort boiling, pasteurisation and sterile filtration ensure that most food borne pathogens do not grow or survive in beer (Bunker, 1955; Donhauser and Jacob 1988, Dowhanick, 1994, Menz *et al*., 2009). In some exceptional cases survival of some food spoilage microorganisms such as *Bacillus cereus* and *Bacillus licheniformis* has been reported in home brewed beer and commercial beer with high pH (4.8-5.0) and alcohol content of 4-5 % ABV (Hankensen and Ziola, 2008).

There is a small range of non pathogenic beer spoilage bacteria which can still survive, grow and spoil beer. Very few cases of beer spoilage have been reported in recent years due to high standards of hygiene and technological improvements within the brewing industry. However, due to the extent of consumer awareness about food and beverage safety and maintaining corporate brand image, beer spoilage microorganisms are of serious concern to breweries worldwide.

The occurrence of beer spoilage organism in different stages of brewing process is shown in Table 2.1. Beer spoilage microorganisms can be broadly classified into Gram positive bacteria, Gram negative bacteria and some wild yeasts. Gram positive beer spoilage bacteria are regarded as the most hazardous for modern breweries (Back *et al*., 2005; Suzuki *et al*., 2008; Suzuki *et al*., 2011); they mainly include lactic acid bacteria belonging to the genera *Lactobacillus* and *Pediococcus* (Rainbow, 1981). Other less

important Gram positive bacteria capable of growth in beer include species belonging to genera *Leuconostoc*, *Micrococcus* and some *Staphylococcus* species (Priest and Campbell, 2003; Vaughan *et al*., 2005). Gram negative beer spoilers mainly include anaerobic bacteria belonging to genera *Pectinatus* and *Megasphaera*. Other significant Gram negative beer spoilers belong to genera *Zymomonas*, *Selenomonas*, *Acetobacter* and *Obesumbacterium.* Certain *Enterobacteriaceae* such as *Rahnella* and *Hafnia* can also spoil beer to some extent (Priest *et al*., 2003; Vaughan *et al*., 2005; Hill, 2009). Wild yeasts in brewing are generally described as yeast strains which are not deliberately introduced and grow uncontrolled in the brewing process (Gilliland, 1971). Wild yeasts are introduced into beer process via pitching yeast, air or other raw materials (Hill, 2009; Vaughan *et al*., 2005). The percentage of beer spoilage incidents reports during the 1980-2002 period is shown in the Table 2.2.

Table 2.1 Occurrence of beer spoilage microorganisms in different stages of the brewing process (adapted from Vaughan *et al.*, 2005)

Stage	Genera	Species
Barley in the field / Malting	*Aspergillus*	*A. fumigates*
	Fusarium	*F. culmorum*
		F. graminearum
Mashing and Wort separation	*Pediococcus*	*P. pentaosaceus*
		P. inopinatus
	Bacillus	*B. coagulans*
	Rahnella	*R. Aquaticus*
	Citrobacter	*C. fruendii*
	Klebsiella	*K. terrigena*
		K. oxytoca
Fermentation	*Wild Yeast*	*Non- Saccharomyces*
		Saccharomyces species
	Pediococcus	*P. inopinatus*
	Selenomonas	*S. lacticiflex*
	Zymophilus	*Z. raffinosivorans*
	Rahnella	*R. aquaticus*
	Obesumbacterium	*O. proteus*
Biological stability and Packaging	*Pectinatus*	*P. cerevisiiphilus*
		P. frisingensis
		P. haikarae
	Megasphaera	*M. cerevisiae*
		M. paucivorans;
		M. sueceinsis

	Lactic acid bacteria	*Lactobacillus* species
		Pediococcus species
		Lactobacillus species
		Pediococcus species
Finished beer	*Pectinatus*	*P. cerevisiiphilus*
		P. frisingensis
		P. haikarae
	Megasphaera	*M. cerevisiae*
		M. paucivorans; *M sueciensis*
	Zymomonas	*Z. mobilis*
	Micrococcus	*M. kristinae*
Beer Dispense	*Acetic acid bacteria*	*A. aceti*
		A. pastorianus
		G. oxydans
	Lactic acid bacteria	*Lactobacillus species*
		Pediococcus species
	Wild Yeast	

Table 2.2 Percentage of beer spoilage incidents reports in Europe during the 1980-2002 period[a]. (adapted from Suzuki, 2011)

Genus/ species[b]	**1980-90**	**1992[c]**	**1993**	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**
L. brevis	40	39	49	38	43	41	51	42	51
L. lindneri	25	12	15	5	4	10	6	13	11
L. plantarum	1	-	-	1	4	2	1	1	2
L. casie/ paracasei	2	3	2	6	9	5	8	4	4
L. coryniformis	3			4	11	4	1	3	6
Ped. damnosus	17	4	3	31	14	12	14	21	12
Pectinatus spp.	4	28	21	6	3	6	5	10	7
Megasphaera spp.	2	7	3	2	2	4	4	4	2
Saccharomyces wild yeasts	N.A[d]	5	5	7	6	11	5	2	3
Non *Saccharomyces* wild yeast	N.A.	0	0	0	3	4	5	0	2
Others	N.A.	2	2	0	1	1	0	0	0

a -This table is adapted from the studies conducted by Back during the 1980–2002 period (Back *et al*., 1994; Back *et al.*, 2003)

b -*L. brevis* includes spoilage incidents due to *L. brevisimilis* (Back *et al*., 1994)

c -In 1992 and 1993 studies, *L. plantarum, L. casei, L. paracasei* and *L. coryniformis* were put together into one group.

d –data not available

2.5 Gram positive beer spoilage bacteria

Most of the Gram positive beer spoilers are lactic acid bacteria, with the most frequently occurring belonging to the genera *Lactobacillus* and *Pediococcus*. These are considered to be the most detrimental due to strong hop resistance and ability to spoil a varied range of beers. The major spoilage effects include formation of haze and ropiness, accompanied with sedimentation, sourness and atypical diacetyl off flavour (Sakamoto and Konings 2003).

2.5.1 Genus*: Lactobacillus*

L. brevis is the most frequently occurring beer spoilage lactic acid bacterium and it is also the most studied *Lactobacillus* species; it is responsible for more than 30-50 % of beer spoilage incidents by the genus *Lactobacillus* (Back, 1988; Back 2005; Hollerova and Kubizniaková, 2001; Suzuki 2011). *L. brevis* prevails in all brewing processes, often isolated from fermenting wort and maturation tanks and it also grows well in a range of LAB (Lactic acid bacteria) detection media compared to hard to grow strains such as *L. lindneri* and *L. paracollinoides* (Suzuki, 2006; Suzuki, 2008a). *L. brevis* is a homofermentative, mesophilic bacterium that grows optimally at 30 ºC and pH 4-6 (Sakamoto and Konings 2003). The beer spoilage strains of *L. brevis* are generally hop resistant. The beer spoilage ability of *L. brevis* varies according to strain and source of isolation (Back, 2005; Suzuki *et al*., 2011) and non brewery isolates of *L. brevis* tend to show very low beer spoilage ability (Nakagawa, 1978; Suzuki, 2008). *L. brevis* contaminated beer shows haze formation, sedimentation, sourness and super attenuation due to the bacterium's ability to utilise dextrin and starch (Lawrence, 1988). *L. brevis* also tends to show loss of beer spoilage ability after repeated sub culturing on media without hop bitterness compounds hence strain level differentiation of beer spoilage ability of these bacteria is significant (Suzuki, 2011). Beer adapted *L. brevis* strains tend to shrink in size compared to non beer adapted strains most probably to reduce the contact surface with antibacterial compounds in beer in order to adapt to the adverse beer environment (Suzuki, 2008).

L. lindneri is another important beer spoilage *Lactobacillu*s; it is physiologically similar *to L. brevis* and has been differentiated from *L. brevis* based on 16S RNA gene sequence as a separate species (Back, 1996). It grows optimally at 30 ºC and pH 4-6 (Sakamoto and Konings 2003) and is highly adapted to hop compounds (Back, 1981; Suzuki, 2006). Thermal resistance of *L. lindneri* is high compared to other beer spoilage

Lactobacilli and it may survive high pasteurisation treatment (Back, 1992). *L. lindneri* grows poorly on many detection media and often causes beer spoilage without being detected in the brewing process (Suzuki, 2008; Suzuki 2011). Beer contaminated by *L. lindneri* forms less haze and sediments compared to other beer spoilage Lactobacilli and there is no noticeable off flavour formation (Back, 2005). *L. lindneri* adapted to the beer environment shows reduced cell size similar to *L. brevis* to survive in the harsh beer environment and it can easily penetrate membrane filters used for removal of contaminants from beer (Suzuki, 2008; Suzuki, 2011). *L. lindneri* also shows ability to adhere to brewing yeast in late fermentation and during the maturation process and can be transferred in the pitching yeast.

Relatively recently *Lactobacillus* species *L. paracollinoides* (Suzuki, 2004) and *L. backi* (Bohak, 2006) were found to show strong beer spoilage ability but the frequency of beer spoilage incidents is not well known. These species are considered unique to the brewery environment (Suzuki, 2011). *L. backi* has been reported to be a contaminant of lager, pilsner and wheat beer (Bohak, 2006). *L. paracollinoides* shows poor growth on many media similar to *L. lindneri* which could be the reason that these species remained uncharacterised until the last decade. Genetically, *L. paracollinoides* and *L. backi* are closely related to *L. collinoides* and *L. coryniformis*, respectively, hence these bacteria may have been misidentified till recent years (Suzuki, 2011). *L. paucivorans*, a novel *Lactobacillus* species was recently proposed by Ehrmann *et al*. (2010). It was isolated from brewery beer storage tanks, but not much is known regarding its beer spoilage ability.

Other heterofermentative *Lactobacillus* species such as *L. casei*, *L. paracasei*, *L. coryniformis*, *L. plantarum* and *L.buchneri* show relatively low resistance to hop bitterness compounds but still can spoil weakly hopped and elevated pH beers. The major spoilage effect is a noticeable off flavour due to formation of diacetyl (Back 2005; Sakamoto and Konings 2003).

2.5.2 Genus *Pediococcus*

Pediococci are Gram positive, homofermentative, cocci shaped bacteria, which typically grow in pairs and tetrads (Dobson *et al.*, 2002). Spoilage of beer is mainly due to acid formation and buttery aroma due to the formation of diacetyl (Jespersen and Jacobsen, 1996). *Pediococci* are found at various stages of beer production from wort till finished beer, mainly in late fermentation and the beer maturation process. There are several *Pediococcus* species which have been isolated from the brewery environment (Suzuki,

2011). Some of the major species are *P. damnosus*, *P. acidilactici*, *P. dextrinicus*, *P. inopinatus*, *P. pentosaceus*, *P. parvulus* and *P. claussenii* (Back and Stackbrandt, 1978; Suzuki, 2011; Dobson *et al*., 2002) but only a few *Pediococci* species are reported to spoil beer. *P. damnosus* is the most common beer spoiler with the ability to produce diacetyl and some strains are reported to produce exopolysaccharides causing ropiness (Back, 2005). *P. inopinatus* and *P. dextrinicus* have been reported to spoil weakly hopped beer with elevated pH (4.2) and low alcohol (Lawrence 1988,). *P. claussenii* has also been reported to spoil beer (Dobson *et al*., 2002)

P. damnosus is similar to *L. lindneri* adhering to brewing yeast which sometimes can cause sedimentation of yeast resulting in fermentation problems (Priest and Campbell, 2003). *P. damnosus* tends to grow slowly on many laboratory media which makes detection of this species difficult using conventional methods (Back, 2005; Taguchi *et al*., 1990).

P. inopinatus has been occasionally isolated from pitching yeast but rarely occurs in other stages (Priest and Campbell, 2003). *P. claussenii* is a relatively recently described beer spoilage species (Dobson *et al*., 2002) and some strains of *P.claussenii* are reported to spoil beer due to production of exopolysaccharides causing ropiness.

2.5.3 Other Gram positive bacteria

Leuconostoc are homofermentative cocci or rod shaped bacteria with similar nutritional requirements to *Lactobacillus* species. *Leuconostoc mesenteroides* has been reported to grow in beer and tends to be highly acid tolerant but this species has not been reported to spoil beer (Priest and Campbell, 2003).

A facultative anaerobic, acid tolerant and hop resistant bacterium, *Kocuria kristinae*, previously known as *Micrococcus kristinae* (Kloos *et al*., 1974), can grow in lower concentration of ethanol and hop compounds and relatively high pH of beer (above 4.5) (Back, 1981). Beer spoilage effects mainly include production of off flavour and fruity aroma (Back, 1981; Sakamoto and Konings, 2003). *Staphylococcus epidermidis* and other *Staphylococcus* species grow poorly at pH below 4.5 and are highly susceptible to hop compounds; hence they are less significant as they are unable to spoil beer (Sakamoto and Konings, 2003; Menz, 2009).

2.6 Other Gram negative bacteria

Only a few Gram negative bacteria have been found to be responsible for beer spoilage. Gram negative, anaerobic beer spoilers belonging to genera *Pectinatus* and *Megasphaera* are regarded as the most important beer spoilage bacteria, mainly in unpasteurised beer. Other Gram negative, anaerobic beer spoilers phylogenetically related to *Pectinatus* and *Megasphaera* belong to genera *Zymomonas*, *Zymophilus* and *Selenomonas*. Gram negative aerobic bacteria have also been reported to spoil beer. Acetic acid bacteria such as *Gluconobacter* and *Acetobacter* are common to breweries but due to effective hygiene and sanitation in modern breweries these bacteria are less important (Sakamoto and Konings, 2003). Certain enterobacteria such as *Hafnia protea* and *Rahnella aquaticus* have been detected in pitching yeast (Hill, 2009).

Previously, acetic acid bacteria such as *Acetobacter* and *Glucanobacter* were important beer spoilers. These bacteria metabolise ethanol to acetic acid giving vinegary flavour to beer (Sakamoto and Konings, 2003) but due to implementation of effective cleaning and sanitation procedures in modern breweries and effective removal of oxygen from post fermentation processes, these bacteria are no longer considered important (Sakamoto and Konings, 2003). *Zymomonas mobilis* is a facultative anaerobe and has been isolated from primed sugars. There has been no report on incidents of spoilage as these microbes utilise only a narrow range of sugars (Sakamoto and Konings, 2003).

Selenomonas lacticiflex is more sensitive to acidic environments than *Pectinatus* and *Megasphaera* and has been isolated from pitching yeast (Suzuki, 2011). It is considered as potential beer spoilage bacterium (Juvonen, 2009). *Z. raffinosivorans* and *Z. paucivorans* have been isolated from pitching yeast but have never been implicated as causative agents for beer spoilage due to their inability to grow in beer (Suzuki, 2011).

2.6.1 Genus: *Pectinatus*

Pectinatus was reported as a new genus of Gram negative, catalase negative, motile, obligate beer spoilage bacteria in the 1970s when it was first isolated from a brewery in the United States in unpasteurized beer stored at 30 $^{\circ}$C (Lee *et al.,* 1978). *P. cerevisiiphilus* was later isolated from breweries in Finland, Germany, Norway, Japan, Spain, Netherlands, Sweden and France (Haikara *et al.,* 1981; Kirchner *et al.,* 1980; Soberka *et al.,* 1988; Takahashi *et al.,* 1983; Hage and Wold, 2003). In an extensive taxonomic study of anaerobic rods isolated from breweries, a second species of the genus *Pectinatus* was identified as *P. frisingensis* (Schleifer *et al.,* 1990).

P. frisingensis differs from *P. cerevisiiphilus* on the basis of growth rate and substrate utilization. *Pectinatus frisingnesis* can ferment, cellobiose, inositol and N-acetyl glucosamine but it cannot utilise xylose and melibiose which can be utilised by *P. cervisiiphilus* (Scheifer *et al*, 1990) In 2006 a third species, *P. haikarae* was identified on the basis of 16S rRNA gene sequence analysis and differences in sugar utilization, catalase activity, antibiotic resistance and temperature tolerance compared to the two previously characterised species (Juvonen and Suihko, 2006). *P. portalensis* was also proposed as a relatively fast growing, coccoid shaped, new species isolated from the waste water treatment plant of a winery (Gonzalez, 2004), but 16S RNA gene sequencing analysis and phenotypical characteristics of *P. portalensis* type strains CECT 5841^T and LMG 22865^T did not validate as a new species and these strains were identified as cocci shaped *Enterococcus faecalis* (Vereecke and Arahal, 2008).

Important characteristics of beer spoilage *Pectinatus* species are shown in Table 2.3 The genus *Pectinatus* currently comprises three brewery related species: *P. cerevisiiphilus* (Lee, 1978), *P. frisingensis* (Schleifer, 1990) and *P. haikarae* (Juvonen and Suihko, 2006). The growth of *Pectinatus* species is accompanied by extensive turbidity and an offensive aroma similar to rotten eggs due to the production of various fatty acids, hydrogen sulphide and methyl mercaptan (Haikara *et al.*, 1981; Lee *et al.*, 1978)

Table 2.3 Characteristics of beer spoilage *Pectinatus* species (adapted from Zhang et al., 2012)

Characterisitcs	***P. cerevsiiphilus***	***P. haikarae***	***P. frisingensis***
Inhabit	spoiled beer	brewery bottling hall	spoiled beer
G+C content (%)	38.6	39.1	38.4
widthx lenght (µm)	0.7-1.0x3-3-0	0.6-0.8x3-50	0.7-0.9x3-50
Temperature (°C)			
range	10-45	15-30	15-37
optimum	30	20-30	30
pH			
range	3.5-8.5	4.0-8.0	3.5-8.0
optimum	6.5	7	6.5
Gram stain	-	-	
catalase activity	-	+	-
Beer spoilage ability	absolute beer spoiler	potential beer spoiler	absolute beer spoiler

All these three species have been isolated from brewery environments and hence the genus *Pectinatus* was considered to be brewery specific. However recently two new species of *Pectinatus* have been recovered from salty pickle waste water: *P. brassicae*, (Zhang *et al*., 2012) and *P. sottacetonis*, (Caldwell *et al*., 2013). *P. brassicae* may be differentiated from other *Pectinatus* species based on high salt tolerance (Zhang *et al*., 2012). The beer spoilage abilities of *P. brassicae* and *P. sottacetonis* are not known.

2.6.1.1 Current phylogenetic status

Previously, Gram negative anaerobic bacteria belonging to the genus *Pectinatus* were affiliated to sub branch *sporomusa* in the family A*cidamincocaceae* of class *Clostridia* (Willems and Collins, 1995; Strömpl *et al*., 1999; Marchandin *et al*., 2003). In 2010, a new class *Negativicutes*, bacteria having a Gram negative cell wall, was proposed within the phylum fermicutes along with a new order, *Selenomonadales* (Marchandin, 2009) which has changed the taxonomic status of the genus *Pectinatus* affiliating it to class- *Negativicutes* (Marchandin, 2009), Order - *Selenomonadales* (Marchandin, 2009), Family-*Veillonellaceae* (Rogosa 1971; Marchandin *et al*. 2009), Genus *Pectinatus* (Lee *et al*., 1978; Schleifer *et al*., 1990; Juvonen and Suihko, 2006; Zhang *et al*., 2012).

P. cerevisiiphilus, even though discovered before *P. frisingensis*, is suggested to be descended from the latter based on cross reactivity experiments of flagella antibodies (Chaban *et al*., 2005). *P. haikarae* which is capable of growing at slightly lower temperature than the other *Pectinatus* species is suggested to be diverged from *P. cerevisiiphilus* as a result of better acclimatisation to brewery environments. *P. haikarae* is also catalase positive unlike *P. cerevisiiphilus* and *P. frisingensis* which may provide better survival in aerobic brewery environments (Juvonen, 2009).

2.6.1.2 Occurrence of *Pectinatus species* within the brewery environment

Most *Pectinatus* species have been isolated from beer and brewery environments but their natural environment and source of contamination are not well understood (Suzuki, 2011). It has been found that several sources of contamination can be identified in the same brewery. *P. cerevisiiphilus* and *P. frisingensis* have been extensively studied and *P. frisingensis* has been more frequently held responsible for beer spoilage incidents compared to *P. cerevisiiphilus* in unpasteurised beer (Motoyama *et al.*, 1998; Hage and Wold, 2003; Haikara and Helander, 2006). Along with unpasteurised beer *Pectinatus* species have also been isolated from drainage systems, water pipe systems, various

equipment in bottling halls, air of bottling halls, conveyors belts and oil lubricants, cracked floors and tiles of the filling hall (Back, *et al*., 1988; Back, 2005; Dürr, 1983; Lee *et al*., 1980; Soberka *et al*., 1988; Motoyama, 2003). *Pectinatus* have also been reported in pitching yeast and CO_2 recovery systems (Haikara and Helander, 2006). The isolation of *Pectinatus* has been mainly from beer filling halls and filling machines and prolonged survival of *Pectinatus* in biofilms formed in beer filling areas indicates that water may be a possible source of contamination (Back, 2005). Viable *Pectinatus* strains, although being anaerobic bacteria, have been found in aerosols around fillers of bottling machines indicating that air or other aerosols around fillers could be a possible source of contamination (Haikara and Helander, 2006). Survival of *Pectinatus* in aerobic environments of beer filling halls can be possible due to formation of biofilms with mixed populations of various microflora commonly occurring in brewery environments (Back, 1994). *P. portalensis* has been isolated from waste water of a winery (Gonzalez *et al*., 2005) and recently *P. brassicae* has been isolated from a pickle waste water plant (Zhang *et al*., 2012), suggesting that occurrence of *Pectinatus* species can be broadened from brewery environments to anaerobic and organic matter rich niches in food production and other beverage production environments.

2.6.1.3 Beer spoilage ability

Brewery related *Pectinatus species* are non spore forming, motile rods with flagella attached laterally to one side of the cells. Young cells show an X shaped pattern formation during movement and old cells show slow snake like movement (Lee *et al.*, 1978; Haikara *et al*., 1981; Schleifer *et al*., 1990; Juvonen and Suihko, 2006).

For *P. cerevisiiphilus* and *P frisingensis* growth occurs between 15- 40 ºC and optimum growth occurs at 30-32 ºC (Lee *et al*., 1978; Schleifer *et al*., 1990; Juvonen and Suihko, 2006). Growth of *P. haikarae* is inhibited at temperatures above 37ºC and optimum growth occurs between 20-30ºC. *P. frisingensis* can maintain cellular homeostasis during sudden changes in temperature (Chihib and Tholazan, 1999). *P. cerevisiiphilus* when co-cultured with *S. cerevisiae* showed growth at 8 ºC and it also affects the growth of *S. cerevisiae* (Chowdhury *et al.*, 1997).

The pH range for growth of these bacteria lies between 3.5 to 8.0 and optimum growth occurs at 6.5-7 (Lee *et al*., 1978; Schleifer *et al*., 1990; Juvonen and Suihko, 2006). *Pectinatus species* can tolerate ethanol concentration up to 3.7 %- 4.4 % (w/v) and growth is completely inhibited at ethanol concentration of 5.5 % (w/v) (Haikara and

Helender, 2006; Haikara *et al*., 1981). *P. cerevisiiphilus* and *P. frisingensis* can grow at a dissolved oxygen concentration of 0.4-0.8 mg/L and *P. frisingensis* showed better tolerance to dissolved oxygen compared to *P. cerevisiiphilus* (Chowdhury *et al*., 1995). The oxygen tolerance of *P. cerevisiiphilus* has been reported to improve with a decrease in temperature (Flahaut *et al*., 2000). *P frisingensis* is better adapted to acidic and thermal environments compared to other *Pectinatus* species (Juvonen, 2009). *P. frisingensis* can metabolise a wider range of fermentable sugars but it cannot utilise ethanol, maltose and essential amino acids (Schleifer, 1990; Tholazan *et al*., 1996).

P. cerevisiiphilus, P. frisingensis and *P. haikarae* are reported to have strong beer spoilage ability mainly in unpasteurised and low alcohol content beer (Haikara and Helender, 2006). The spoilage effects mainly include production of propionic acid, acetic acid, H_2S, dimethyl sulphide (DMS), and methyl mercaptan. The rapid cell growth makes beer turbid and beer typically smells like rotten eggs due to production of sulphur compounds (Haikara and Helender, 2006; Juvonen, 2009).

2.6.2 Genus Megasphaera

Genus *Megasphaera*, originally described by Rogosa (1971), consists of five validly published species; *M. elsdenii* (Rogosa, 1971), *M. cerevisiae* (Engelmann and Weiss, 1985), *M. micronuciformis* (Marchandin *et al*., 2003), *M. paucivorans* and *M. sueciensis* (Juvonen and Suihko, 2006). *Megasphaera* species have been isolated from a variety of different environments such as human clinical specimens, rumen gut flora and brewery environments (Marchandin *et al*., 2003; Zozaya-Hinchliff *et al*., 2008).

Important characteristics of beer spoilage *Megasphaera* species are shown in Table 2.4. At present the genus *Megasphaera* is comprised of three brewery associated species. *Megasphaera cerevisiae*, originally described by Engelmann and Weiss, (1985) was the first brewery associated species, mainly representing low- alcohol beer spoiling cocci. *M. cerevisiae* was responsible for 3-7 % of beer spoilage cases in Europe during the period 1980 to 2002, mainly in non pasteurised beer (Back *et al.*, 1988; Back, 1994). Later, two novel coccoid shaped bacteria were identified associated with beer spoilage and named *M. paucivorans* and *M. sueciensis* (Juvonen and Suihko, 2006). Spoilage effects of *M. cerevisiae* include turbidity and unpleasant odour, due to production of H_2S and short chain fatty acids. All *Megasphaera* species related to the brewery environment are strictly anaerobic, Gram negative, non spore forming and non motile (Engelmann and Weiss., 1985; Juvonen and Suihko, 2006).

Table 2.4 Characteristics of beer spoilage *Megasphaera* species (adapted from Juvonen and Suihko, 2006)

Characterisitcs	*M. cerevisiae*	*M. paucivorans*	*M. seuciensis*
Inhabit	spoiled beer	spoiled beer	spoiled beer
G+C contnent	42.4-44.8	40.5	43.1
Width x lenght (µm)	1.5-2.1	1.2-1.9 x 1-1.4	1-1.4 x 0.8-1.2
Temperature (°C)	10-37	10-30	10-30
pH range	4-8	4-8	4-8
Gram stain	-	-	-
catalase activity	-	-	-
Beer spoilage ability	4.2 % ABV, pH < 4-4.1, 33-38 IBU	2.8-5 % ABV, pH 4.3, 33-38 IBU	2.8 % ABV, pH 4.9, 33-38 IBU

2.6.2.1 Current phylogenetic status

Similar to *Pectinatus*, the genus *Megasphaera* was previously affiliated to sub branch *sporomusa* in the family *Acidamincocaceae* of class *Clostridia* (Rogosa *et al*., 1971; Engelmann and Weiss, 1985; Marchandin *et al*., 2003; Juvonen and Suihko, 2006). In 2010, the taxonomic status of the genus *Megasphaera* was changed affiliating it to class- *Negativicutes* (Marchandin, 2009), Order - *Selenomonadales* (Marchandin, 2009), Family-*Veillonellaceae* (Rogosa 1971; Marchandin *et al*. 2009).

2.6.2.2 Occurrence of *Megasphaera* within the brewery environments

Brewery related *Megasphaera* species share common ecological niches with *Pectinatus* but are less wide-spread (Seidel *et al*., 1979; Haikara and Helander, 2006; Juvonen, 2009; Suzuki, 2011). *M. cerevisiae* has been extensively studied as a contaminant of unpasteurised beer. M. *cerevisiae* has also been reported from brewery bottling hall biofilms and occasionally from pitching yeast and CO_2 recovery systems (Haikara and Helander, 2006). Occurrence of M. *paucivorans* and M. *sueceinsis* has not been studied well but these species have been reported to be isolated from unpasteurised beer and other brewery environments (Juvonen, 2009).

2.6.2.3 Beer spoilage ability

Growth occurs in temperature range 15-37 ºC and optimum growth is reported to be at 28 ºC (Haikara and Lounatmaa, 1987). No growth is observed at 10 and 45 ºC (Juvonen and Suihko, 2006). *Megasphaera cerevisiae* is limited to ethanol concentration of 2.1 % (w/v) and its growth completely inhibited at a concentration of 4.2 % (w/v) (Haikara and Lounatmaa, 1987. Growth at normal beer pH has been detected but its growth completely inhibited at pH 4.1 and above (Haikara and Helender, 2006).

Beer spoilage ability of *Megasphaera* species is not extensively studied compared to *Pectinatus* (Juvonen, 2009). *Megasphaera* species mainly affect low alcohol and unpasteurised beer producing turbidity and metabolic products such as butyric acid and minor amounts of acetic acid, valeric acid, caprioc acid and acetoin (Seidel *et al*., 1979). Considerable amounts of H_2S are produced in spoiled beer giving very unpleasant odour (Haikara and Lounatmaa, 1987; Lee, 1994).

2.7 Microbial detection methods in breweries

Quality of packaged beer is assured by taking preventive measures during beer production and packaging stages. The main objective of implementing microbial detection methods is to verify assurance of microbial quality of the final product and effectiveness of cleaning and sanitation processes during beer production. There have been extensive studies on microbial detection methods in recent years (Priest, 2003; Hill, 2009; Strogards *et al*., 2006). There are no official data regarding microbiological specifications in the brewing process. The acceptable detection level specifications suggested by Jespersen and Jakobsen (1996) are shown in Table 2.5 However, even a low level of contaminants poses a potential risk of spoilage due to the intended shelf life of packaged beer (Juvonen, 2009).

Conventional detection in breweries relies on cultivation of beer spoilage microorganisms on selective media followed by microscopic examination and sensory analysis. Rapid methods are mainly based on visualisation of cells and micro-colonies and analysis of cellular constituents and genetic material (Juvonen, 2009).

2.7.1 Conventional methods

Conventional microbiological detection methods are based on selective staining followed by microscopic analysis and simple biochemical and physiological analysis methods such as nutrient assimilation, selective growth on specific agar and sensory evaluation (Hill, 2009; Juvonen, 2009).

Conventional methods involve collection of samples in various forms such as direct beer or rinse sample, yeast slurry or swab samples from various vessels. Microorganisms from the samples are further concentrated either through filtration or culture enrichment in specific media. For direct beer samples, 100-500 ml of beer is membrane-filtered and plated on selective or non selective media. The recovery rate of *Pectinatus* and *Megasphaera* was low using membrane filtration hence; this method cannot be effectively used for obligate anaerobes (Haikara, 1985).

Other conventional methods include shelf life testing by incubation of packaged beer at room temperature for the specific period of 4-6 weeks followed by microbiological analysis of beer using an agar plating method or sensory evaluation of off flavour formation. The forcing test involves incubation of packaged beer with concentrated media to enhance growth of spoilage microorganisms followed by detection of haze and turbidity formation (Haikara and Helender, 2006).

Various selective and non selective media are used for culture enrichment of the beer for microbiological detection (Hill, 2009). The most common bacterial detection media are listed in the Table 2.6. In addition to these, various media such as Schwartz differential medium (SDM) (Brenner, 1970), Lin's wild yeast medium (LWYM) with 200 ppm $CuSO_4$ and WLN (Wallerstein Laboratories Nutrient) medium are available for detection of wild yeast contamination (Jespersen and Jacobson, 1996).

Table 2.5 Acceptable limits of detection of beer spoilage microorganisms in the brewery

Samples	Sensitivity
Cold aerated wort	1 microorganism per 25 ml
Pitching yeast	1 bacterium per ml and 1 wild yeast per 10^6 culture yeast
Fermenting wort	1 organism per ml
Tank bottoms	1 organism per ml
Beer in storage	1 organism per ml
Filtered beer	1 beer spoilage organism per 100 ml or 10-10^2 non beer spoilage microorganisms per 100 ml
Packaged beer (non pasteurised or flash pasteurised)	10-10^2 non beer spoilage organisms per 100 ml
Rinse water (end of cleaning in place)	1 organism per 100 ml

Source : Jespersen and Jakobsen, (1996)

Table 2.6 Culture media for the detection of beer spoilage bacteria

Media	Bacteria	Recommendation[3abc]
MRS (de Man Rogosa and Sharpe)	LAB[1],	EBC[a], ASBC[b], BCOJ[c]
Raka Ray	LAB, G(-)[2]	EBC, ASBC, BCOJ
VLB S-7 (Versuchs- und Lehranstalt fuer Brauerei in Berlin)	LAB	EBC, BCOJ
HLP (Hsu's *Lactobacillus* and *Pediococcus* medium)	LAB	EBC, BCOJ
WLD (Wallerstein Differential)	LAB	EBC, BCOJ
Nakagawa medium	LAB	EBC, BCOJ
SDA (Schwartz Differential agar)	LAB	EBC, BCOJ
Concentrated MRS	G(-)	EBC, BCOJ
PYF (Peptone-Yeast extract- Fructose)	G(-)	EBC, BCOJ
Thioglycolate medium	G(-)	EBC
LL (lead lactate) agar	G(-)	EBC
UBB/UBA (Universal Beer broth/agar)	LAB, G(-)	EBC, ASBC, BCOJ
KOT (Kirin-Okhochi-Taguchi) medium	*Pediococcus*	
NBB (Nachweis medium für Bierschädliche Bakterien)	LAB, G(-)	EBC, BCOJ
Brewer's tomato juice Medium	LAB, G(-)	ASBC
LMDA (Lee's Multi Differential Agar)	LAB	ASBC
BMB (Barney-Miller- brewery medium)	LAB	ASBC
SMMP (Selective Media for *Megasphaera* and *Pectinatus*)	G(-)	ASBC,
ABD (Advanced Beer Detection media)	LAB[4]	BCOJ
S.I (Sugama- Iguchi) medium	LAB	-
Kunkee medium	LAB	-
MRS agar or broth with mevalonic acid supplemented MRS (S-MRS)	LAB	-
Pediococcus damnosus medium (PDM)	*Pediococcus*	-

1- Lactic acid bacteria, mainly *Lactobacillus* and *Pediococcus*
2- Gram negative beer spoilage bacteria (both aerobic and anaerobic)
3- 3a- EBC- European brewing convention
3b- ASBC- American Society for Brewing Chemists
3c- Brewing Convention of Japan
4 - ABD medium is mainly used for detection of hard to culture LAB medium.

Source- Sakamoto and Konings, 2003; Suzuki, 2008; Suzuki, 2011

It has been observed that growth of microorganisms in beer varies significantly not only at species level but also at strain level due to adaptation to particular beer environments. In addition very low levels of microbial contamination make culture enrichment of these microorganisms very difficult and they often remain undetected. Conventional microbial detection requires long incubation times and membrane filter based methods are not sensitive enough to detect anaerobic beer spoilage bacteria (Haikara, 1985) which often leads to incomplete and misleading results. As a consequence, more sensitive and rapid detection methods have gained importance in recent years.

2.7.2 Physical analysis based methods

Physical analysis based bacterial detection involves isolation and characterisation of microorganisms based on physical parameters such as measurement of absorbance, turbidity, impedance and conductance of wort and beer samples. Other procedures include calorimetric, flow cytometry and micro-colony methods (Priest and Campbell, 2003; Hill, 2009).Spectrophotometry and turbidometry methods are commonly used in food, dairy and brewing industries (Priest and Campbell, 2003). Automated turbidometry has been previously described in detection of bacterial contamination in brewing yeast (Haikara, 1990).

The micro-calorimetry method is based on measurement of heat fluctuation. As most of the metabolic activities during growth of bacterial contaminants are exothermic this can cause changes in the thermal properties of the samples. Even a small fluctuation in temperature can be measured using a micro calorimeter which provides valuable data regarding microbial growth. Microcalorimetry based methods are often used in clinical applications but use in breweries is limited (Hill, 2009).

Flow-cytometry methods are based on analysis of light scattering properties of a laser beam which is passed through a uniform flow of rapidly moving samples containing cells or any other particulate material. It is an advanced automated method which provides quantitative data regarding cell shape, size, number and granularity (Melamed *et al*., 1990; Shapiro *et al.,* 1995). Use of various fluorescent staining dyes capable of staining cellular components such as DNA and cell wall proteins can also provide a count of viable and non viable cells. Flow cytometry based methods are highly sensitive but it is also a relatively slow process and analysis can be interfered with by small particulate materials. Flow cytometry has been utilised for detection of brewery related *Lactobacillus* species (Bunthof *et al*., 2001) and wild yeast (Jespersen *et al*., 1993).

Micro-colony and Direct epi- fluorescence filter technique (DEFT) are methods based on selectively staining cells collected on a filter membrane. The micro-colony method requires incubation of filtered cells typically for 24 hours to form invisible micro-colonies before staining and detection but DEFT does not require any incubation. DEFT methods involve use of nucleic acid staining with acridine orange, hence staining can distinguish between viable and non viable cells. A micro colony based method using carboxyfluorescein diacetate (CFDA) stain for detection of slowly growing beer spoilage lactic acid bacteria has also been described (Asano *et al*., 2009).

2.7.3 Biochemical analysis based methods

ATP bioluminescence based hygiene detection methods are routinely used in the food process industry (Shama and Malik, 2012) and beverage industries (Strogärds, 2000; Hill, 2009). They are quicker and cost effective in comparison with conventional detection methods. ATP molecules are produced in abundance in viable cells but the concentration starts to deplete in non viable cells. ATP bioluminescence is a luminescence based method; ATP reacts with luciferin-luciferase enzyme complex emitting light which can be measured using a luminometer. The amount of light emitted (Relative Light Units- RLUs) is proportional to the level of ATP (Shama and Malik, 2012), which can be further correlated to concentration of viable cells.

There are various commercial based hygiene monitoring systems are available for analysis of varied samples from the food and beverage industries. Micro star™, Bev-Trace™, Aqua-trace™, 3M Clean-trace™, Spot check™, Pro-clean™ are some of the examples.

2.7.4 Protein analysis

Analysis of cellular proteins by separating them based on size and ionic charge using polyacrylamide gel electrophoresis (PAGE) can provide distinctive patterns of protein fingerprints which are unique to given species or strain of microbes. The study of protein fingerprints can be used to differentiate microbial contaminants at strain level. PAGE has been implemented to characterise the relationship between beer spoilage ability of *L. brevis* and cellular proteins such as S layer protein (Yasui *et al.,* 1995) and D-lactate dehydrogenase (Takahashi *et al.,* 1999).

2.7.5 Immunoassay based methods

Immunoassays are based on use utilisation polyclonal antibodies to detect and characterise beer spoilage bacteria (Russell and Stewart, 2003). Anaerobic beer spoilage bacteria *Pectinatus* and *Megasphaera* have been characterised and detected using antibodies and synthetic peptides in various studies (Ziola *et al*., 2000a; Hakelehto, 2000; Haikara and Helander, 2006). An immuno- fluorescence filter assay has also been reported for detection of *Pectinatus cerevisiiphilus* (Gares *et al*., 1993). Differentiation of *Pediococcus* and *Lactobacillus* using monoclonal antibodies has been documented (Whiting *et al*., 1992; Ziola *et al.*, 2000b and Tsuchiya *et al.*, 2002). Detection and enumeration of beer spoilage lactic acid bacteria using monoclonal chemiluminescence enzyme immunoassay and a charge coupled device (CCD) camera has also been described (March *et al.*, 2005).

2.7.6 Chromatography based methods

Bacterial contamination is mostly accompanied by production of metabolic products such as volatile and non volatile organic acids. Their concentration can be determined by gas chromatography, high performance liquid chromatography and other chromatographic techniques. Various commercial techniques are available for detection and partial characterisation of bacteria using chromatography analysis (Priest and Campbell, 2003). Metabolite analysis of *Pectinatus* and *Megasphaera* cultures has been previously reported using gas chromatography (Haikara and Helander, 2006). Gas chromatography analysis of cellular fatty acids of *Pectinatus* and *Megasphaera* has also been documented (Helander *et al*., 2004). Cellular fatty acids have been used previously as cellular markers for identification of microbial species in biofilms from brewery bottling plants (Timke, 2005).

Gas chromatography analysis of cellular fatty acids has been utilised for characterising bacterial species belonging to sub class *sporomusa* (Moor *et al.*, 1994). In routine quality assurance of beer, gas chromatography analysis is utilised to monitor the diacetyl to pentanedione ratio in fermenting wort and bright beer as an elevated ratio can be due to diacetyl producing contaminants such as *Pediococcus* and *Lactobacillus* (Lodolo *et al*., 2008). These microbes take weeks to be detected on microbial media and some hard to grow strains are often undetected (Suzuki, 2011).

2.7.7 Nucleic acid analysis based methods

Nucleic acids (both DNA and RNA) have been extensively studied and applied to analysis in the food and beverage industries (Mozola, 2000). Probes and primers based on the 16S rRNA gene have been widely and extensively used as phylogenetic markers for identification and characterisation of beer spoilage microorganisms (Juvonen, 2009). There is a vast amount of information on bacterial 16S rRNA gene sequences in nucleotide databases which can be used for development of highly specific nucleic acid based detection and characterisation methods (Yasuhara, 2001).

2.7.7.1 Molecular probes

Nucleic acid probes are short sequence oligonucleotides of DNA or RNA specific for target genus or species which are labelled with a radio-labelled molecule, fluorescent dye or luminescent molecules. The hybridisation of these probes with target nucleic acid is carried out in either *in-vitro* or *in-situ* under highly stringent conditions to eliminate unspecific binding. The unhybridised probes are removed by washing and intensity of the hybridised probe is detected with an appropriate detector which gives a direct measure of target nucleic acid present.

FISH (Fluorescent *in-situ* hybridisation) methods have been widely researched as a probe based procedure for detection and characterisation of brewery contaminants. The first application of FISH reported for detection of *Pectinatus cerevisiiphilus* and *P. frisingensis* used fluroscein thiocyanate labelled DNA probes (Yasuhara *et al.,* 2001). Application of FISH has been reported for detection of *Fermicute* brewery contaminants (Meier *et al.,* 1999) and characterisation of microbial communities of a brewery hall (Timke *et al.,* 2005). A recent application involves detection of beer spoilage *Lactobacillus* using FISH in combination with a micro colony method for detection and enumeration of *L. brevis* (Meng *et al.,* 2012). Real time PCR in combination with micro array has been reported for detection of viable bacterial cells using fluorescent labelled DNA probes targeting the 16S-23S RNA spacer region (Weber *et al*., 2008).

2.7.7.2 Automated ribotyping

Ribotyping involves amplification of bacterial ribosomal genes followed by selective fragmentation of DNA by one or more restriction enzyme treatments. The ribotyping method has been used for characterisation and differentiation of novel beer spoilage *Pectinatus* and *Megasphaera* species from existing ones (Juvonen and Suihko, 2006). Automated ribotyping has also been utilised for detection of common brewery

contaminants such as *L. lindneri* and *Pectinatus* species (Braney *et al*., 2001). An automated ribotyping with PCR based method has been described to characterise brewery strains (biotype-1) of *O. proteus* (Koivula *et al*., 2006).

2.7.7.3 Polymerase chain reaction based methods

The polymerase chain reaction originally described by Mullis *et al*., 1986 is one of the most extensively used molecular techniques. PCR is basically *in-vitro* amplification of target DNA fragments by repeated denaturation of DNA fragments, annealing of specific oligonucleotide primers with complementary sequences of DNA and extension of the DNA fragment using DNA dependent DNA polymerase (Mullis *et al*., 1987; Mullis and Faloona, 1987). PCR has been widely applied in identification and characterisation of bacteria in the food and beverage industries (Russell and Stewart, 2003).

In end point PCR, amplification is carried out first and products are examined after amplification is completed, typically by running amplified products on agarose or polyacrylamide gels and DNA is visualised using DNA staining agents such as ethidium bromide or SYBR- green (Mackay, 2004; MacKay *et al*., 2007). Multiplex PCR is a modification of conventional PCR where more than one target molecule is simultaneously amplified using more than one set of primers. Quantitative PCR is a relatively recent approach where the target DNA fragment is detected and quantified as the reaction proceeds. A summary of PCR based methods applied in detection of brewery contaminants is given in Table 2.7.

Table 2.7 Summary of PCR based methods used for detection of brewery contaminants (adapted from Juvonen, *et al*., 2009)

PCR technique	*Bacteria*	Target gene	Application	References
EP PCR	*L. brevis*	5S rRNA	Beer	Tsuchiya *et al*., 1992
EP PCR	*L. brevis*	5S rRNA	Beer	Tsuchiya *et al*., 1993
EP PCR	*Lactobacillus*	16S rRNA	Beer	Di Michele and Louis, 1993
EP PCR	*Lactobacillus*	16S rRNA	Pure cultures	Taguchi *et al*., 1995
Nested EP PCR	*Lactic acid bacteria*	16S rRNA	Yeast slurry	Stewart and Dowhanick, 1996
EP PCR	*Lactobacillus*	Hor A	Pure cultures	Sami *et al*., 1997
EP PCR	*L. lindneri*	16S rRNA	Pure cultures	Yasui, 1997
EP PCR	*Lactobacillus species*	16S rRNA	Pure culture	Sakamoto, 1997
EP PCR + CH	*Pectinatus, Megasphaera*	16S rRNA	Beer	Satokari *et al*., 1998
EP PCR	*Lactobacillus, Pectinatus and Megasphaera*	16S rRNA	Beer	Juvonen and Satokari, 1999
EP PCR	*L. paracollinoides*	16S rRNA	Pure cultures	Suzuki *et al*., 2004
EP PCR	*Lactic acid Bacteria*	Hor B, Hor C	pure cultures	Suzuki *et al*., 2005
RT- PCR	*Lactobacillus, Pediococcus, Pectinatus and Megasphaera*	16S r RNA	Beer	Keinhe *et al*., 2005
EP PCR, RT PCR	*O. proteus (biotype-I)*	16S rRNA	Beer	Koivula *et al*., 2006
RT- PCR	*Lactic acid bacteria*	Hor A	Pure cultures	Suzuki *et al*., 2006,

multiplex EP PCR	*Lactic acid bacteria*	hit A, hor A, hor C, ORF5, 16S rRNA	Pure cultures	Hankensen 2008
multiplex PCR / SAPD	*Pediococcus*	23S rRNA	Pure Cultures	Pfannebecker *et al*., 2008
PCR –RFLP/ RT PCR	*Clostridia- beer spoilers*	16S RNA	Pure cultures Brewery samples	Juvonen *et al.,* 2009
multiplex RT- PCR	*Firmicutes*	16S rRNA	beer	Hankensen 2008b
multiplex EP PCR	*Lactobacillus, Pediococcus, Megasphaera and Pectinatus*	16s rRNA , ITS	Pure cultures	Asano *et al*., 2008, Ijima *et al.,* 2008
EP PCR- RFLP	*Megasphaera*	16S r RNA	Pure cultures	Ohnishi *et al.,* 2011

1. EP- PCR – end point polymerase chain reaction
2. CH- colorimetric hybridisation
3. RT- PCR- real time polymerase chain reaction
4. RAPD- restriction fragment length polymorphism
5. SAPD- specific amplified polymorphic DNA

2.8 Hybridisation Protection Assay

Nucleic acid hybridisation is based on the ability of denatured nucleic acid to reanneal with its complementary strand and retain its duplex structure in an environment just below the melting temperatures (T_m) (Wetmur, 1991). The nucleic acid hybridisation could occur between DNA-DNA, DNA-RNA and RNA-RNA strands. One of the complementary strands of DNA or RNA can be labelled with isotopic or non isotopic molecules and this can be exploited to measure affinity and specificity of hybridisation duplexes (Frier *et al*., 1997). Rapid and accurate measurement of hybridisation of a labelled nucleic acid probe with its complementary sequence has been the basis for developing various hybridisation methods applied in mechanistic, diagnostics and therapeutic usage (Mazumdar *et al*., 1998). Nucleic acid hybridisation methods have also been extensively used in identification of microbial contaminants in food and beverages industries (Mozola, 2000).

The nucleic acid hybridisation based methods can be broadly classified as either heterogeneous or homogenous assay. The heterogeneous assay involves physical separation of unhybridised probes from hybridised probes. Membrane bound hybridisation assays such as Southern blots (Southern, 1975), Northern blots (Alwine *et al*., 1977) and physical separation methods including gel electrophoresis are some examples of heterogeneous assays. These assays are intensive, time consuming and incapable of direct monitoring of the hybridisation (Mazumdar *et al*., 1998).

In homogenous hybridisation assays, hybridised and unhybridised nucleic acids exhibit distinguishable properties, which make their separation unnecessary for analysis. Homogenous hybridisation assay formats such as FISH (fluorescence in situ hybridisation) (Yasuhara *et al*., 2001), fluorescence labelled DNA probe based microarray (Weber *et al*., 2008), calorimetric hybridisation (Satokari *et al*., 1998) and real time PCR (Juvonen, 2009) have been applied in detection of beer spoilage microorganisms.

The conventional hybridisation based assays were based on utilisation of radioactive label. There are major concerns about using radioactive labels such as limited shelf life, safety and disposal issues (Nelson and McDough, 1990). Extensive effort has resulted in development of non radioactive labelling techniques such as colorimetric, chemiluminescent and fluorescent assay formats (Mansfield *et al*., 1995).

Chemiluminescence can be simply explained as the production of energy in the form of light via chemical reaction (Weeks, 1983). Today chemiluminescence based analytical techniques are well understood and are extensively used in clinical, environmental, food and pharmaceutical sciences (Roda *et al.,* 2012). Chemiluminescence reaction based assays are highly sensitive, safe and yield quantitative data. Numerous types of chemiluminescent labels are reported such as luminal derivatives, acridinium compounds and their derivatives, coelenterazine and its synthetic derivatives, dioxetanes and their analogues (Dodeigne *et al.,* 2000). Chemiluminescent based applications have also been reported in various formats such as immunoassays, receptor assays, DNA labelled probe based hybridisation and biosensors (Dodeigne *et al*., 2000).

2.8.1 Acridinium esters

The synthesis of the acridinium ester (AE) molecule has been previously described by Weeks *et al.,* (1983) and the mechanism of chemiluminescence of AE has also been well described (McCapra, 1985). AE displays chemiluminescence characteristics when it reacts with alkaline peroxide to yield light with a peak wavelength around 440 nm, which can be measured using any standard luminometer (McCapra, 1976). The chemiluminescent properties of AE are mainly dependent on the nature of the functional group (R group) attached to the acridinium ring (McCapra, 1976). The phenyl esters derivatives of acridinium ester which contain a N-hydroxysuccinimide (NHS group) have been reported in various immunoassays (Weeks *et al*., 1983). The NHS group provides a site for covalent attachment of AE to a primary amine providing effective labelling of primary amine containing compounds. The structure and mechanism of AE NHS ester chemiluminescence is illustrated in Figure 2.5.

2.8.2 Mechanism of chemiluminescence of acridinium ester

Some of the important chemiluminescent compounds such as luminol, lucigenin and acridinium ester react with hydrogen peroxide but the reactions of luminol and lucigenin are catalysed by metal ions whereas reaction of acridinium ester does not require a catalyst (Dodeigne *et al*., 2000).

The mechanism of chemiluminescence reaction with hydrogen peroxide in alkaline solution has been well studied (McCapra and Richardson, 1964; McCapra, 1976).

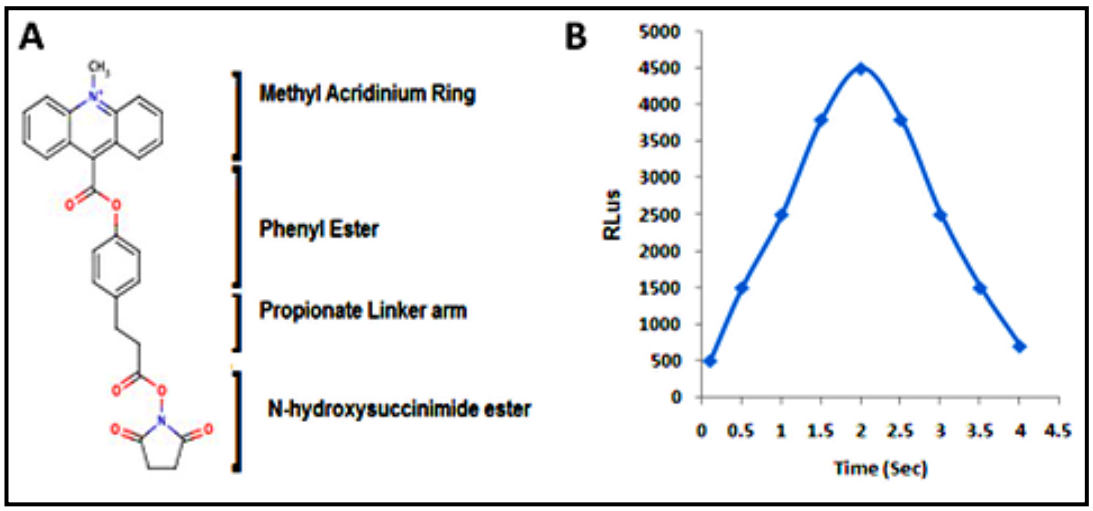


Figure: 2.5: A chemical structure of acridinium NHS ester; B- chemiluminescence reaction of acridinium NHS ester with alkaline peroxide (Source, Nelson *et al.*, 1995)

The probable and other alternative mechanisms of chemiluminescence are shown in Figure 2.6 and routes of the chemiluminescent reaction through pseudo base formation are illustrated in Figure 2.7. According to the most probable mechanism, AE reacts with hydrogen peroxide in the presence of strong base to form intermediate compounds. Dioxetanone formed as an intermediate, ultimately forms an excited state N-methylacridone which emits light concentrating around 440 nm. In strong alkaline solution (pH 12-13) the acridinium ester intermediates are in equilibrium with non chemiluminescent pseudo base. Hence to minimise chemiluminescence loss due to the pseudo base formation, hydrogen peroxide is added in an acidic medium to convert pseudo base to acridinium ester and a strong base is then added to trigger a rapid increase in the pH (pH 12-13) of the medium to initiate the chemiluminescence reaction. As the chemiluminescence lasts for 2-5 seconds, usually flash types of luminometer with dual injectors are used for the measurement of the chemiluminescence reaction. The detection limit of acridinium ester is estimated to be 5x 10^{-19} moles due to the fast kinetics of reaction and short measurement time contributes to lower background noise (Nelson *et al*., 1995).

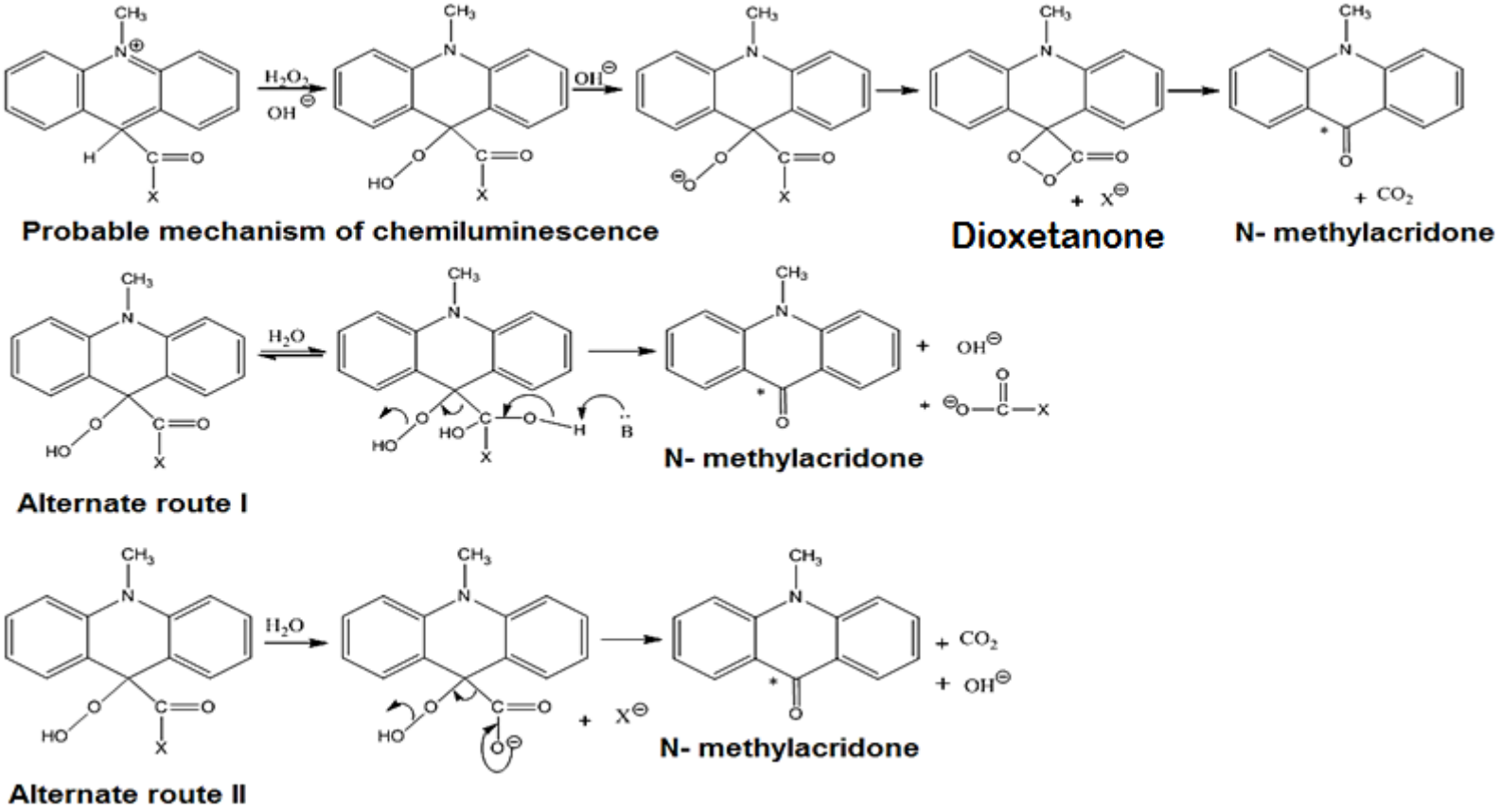


Figure 2.6 - The probable and other alternative mechanisms of chemiluminescence obtained from AE (Dodeigne *et al*., 2000).

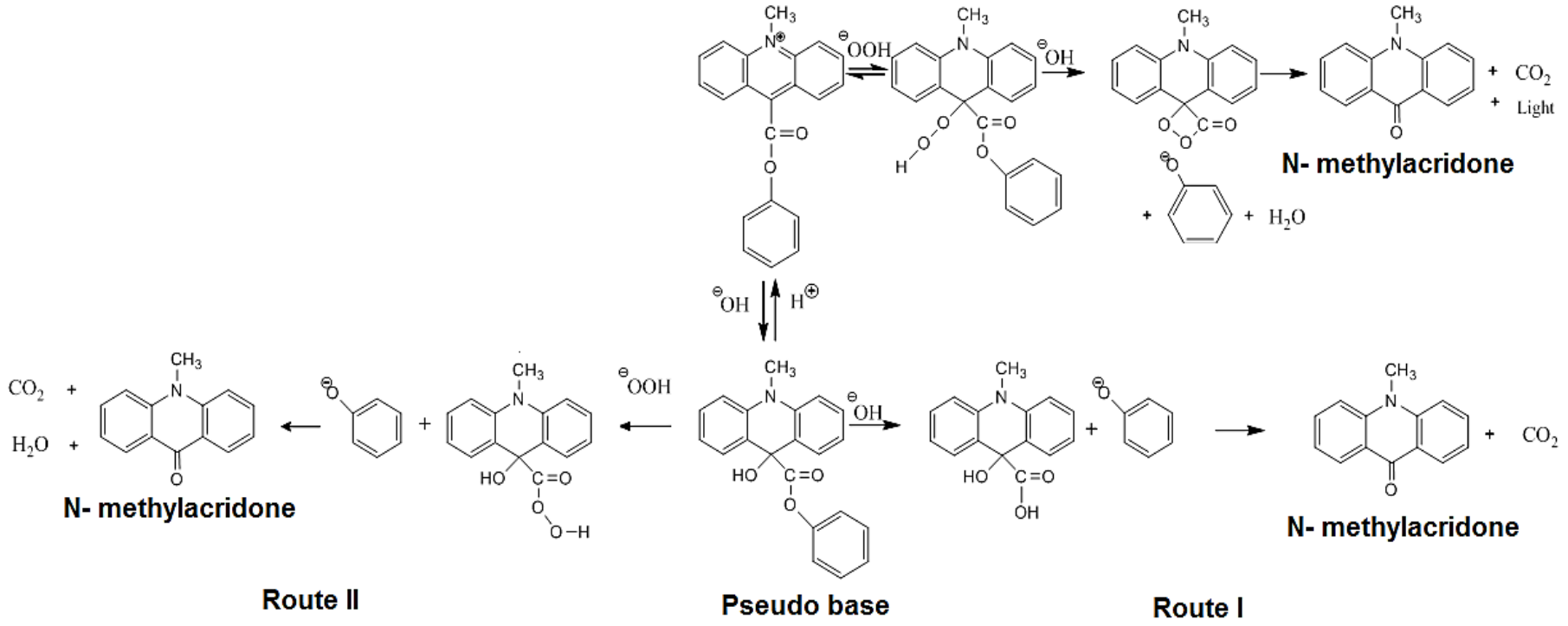


Figure 2.7 - AE chemiluminescent reaction through pseudo base formation and probable alternative routes (Dodeigne *et al.*, 2000).

2.8.3 Working principles of HPA assay

Acridinium NHS esters can be covalently attached to primary amine containing compounds such as amino acids and proteins. In the case of oligonucleotide probes, an amine linker arm can be inserted at various places during synthesis (Arnold *et al.*, 2000) and an oligonucleotide can be labelled with AE without any changes in its chemiluminescence property making it suitable for use in hybridization assays in various formats.

The hybridization protection assay (HPA) format involves hybridization of AE labelled probe with target nucleic acid, followed by a differential hydrolysis step which involves alkaline hydrolysis of free and unhybridised AE probes while the hydrolysis of hybrid AE probe is prevented. The final step involves measurement of the chemiluminescence signal, which is directly associated with the hybridized probes. A simple in-solution protocol, high sensitivity, specificity and versatility are the main advantages of the HPA assay (Nelson *et al.*, 1998). The acridinium ring gets separated from the DNA probe before the chemiluminescence reaction which helps in increasing the sensitivity by minimising intermolecular quenching (Weeks *et al.*, 1983).

2.8.4 Hybridisation Protection Assay formats

Several homogenous formats (hybridisation assay without washing and separation steps) of the HPA assay have been commercially developed which utilise AE labelled probes for detection of RNA or DNA as a target molecule (Granto *et al*., 1989; Tenover *et al*., 1990).

A simple HPA assay is performed in single tube; the target molecule is hybridised with AE probe, followed by hydrolysis of unhybridised AE probes and a final step involves measurement of chemiluminescence. PCR amplified fragments need an additional denaturation step being before subjected to HPA assay.

The alternate format of HPA assay has also been described using magnetic separation. After hybridisation and differential hydrolysis magnetic, amine microsphere beads are used to capture hybridised molecules and used for the detection step. The separation of hybridised probes from unhybridised molecules reduces background noise increasing the assay sensitivity (Nelson *et al*., 1995)

2.8.5 Advantages and disadvantages of HPA assay

HPA assays are homogenous; hence the major advantage of the HPA assay is that there is no need for separation of hybridised molecules from free and unhybridised molecules. HPA assays are highly sensitive due to the rapid chemiluminescent reaction and low background noise. HPA assays have been described to differentiate between target molecules with a single mismatch (Nelson *et al*., 1998; Nelson and Kacian, 1990; Nelson *et al*., 1996; Goto *et al*., 2002), hence the assay can be utilised with high specificity. HPA assay formats are simple and rapid, and the entire assay can be performed within 1-2 hours hence a large number of samples can be processed within a short period of time. The data available for HPA assay are quantitative, reliable and reproducible hence the results are easy to analyse. The HPA assay can be applied to the detection of DNA and RNA molecules from diverse sources; hence these assay formats are versatile and show compatibility for use in clinical, pharmaceutical, food and beverage industries.

The major disadvantage of the HPA assay is that the AE molecule is only stable within a specific pH and temperature range (Nelson and Kacian, 1990). The AE molecule cannot be utilised for probe labelling using a PCR reaction due to thermal instability whereas other molecules used in chemiluminescent based assays such as digoxygenin can be used to produce DIG-labelled amplified fragments. The AE chemiluminescent reaction is irreversible so the sample cannot be revived and re-analysed (Arnold and Nelson, 1999). Inherent chemiluminescence of nucleic acid samples can increase the background during HPA assays and can potentially decrease the overall assay sensitivity (Nelson *et al*., 1995). The background noise can be eliminated using additional processing of samples which could result in an increase in time required for the analysis.

2.9 Application of HPA assay

The HPA assay has been widely used in clinical laboratories for detection of pathogens (Clancy *et al*., 2012; Marlowe *et al*., 2003; Harper and Johnsons, 1990). It has also been used for detection of target amplified products from PCR (Mullis and Faloona, 1987; Nelson and McDonough, 1990) and in genetic mutation studies (Nelson *et al*., 1998; Dhingra *et al*., 1991). Applications of the HPA assay in basic nucleic acid research such, as studies of the DNA double helix (Beckar *et al*., 1999; Majlessi and Becker, 2008) and nucleic acid hybridization kinetics (Mazumdar *et al*., 1998) have also been documented.

The HPA assay has been utilized for detection of *Listeria monocytogenes* from contaminated food samples (Clancy et al., 2012) but application of the HPA assay is limited in the food industry (Mozola, 2000). For the present study HPA formats were developed for detection of beer spoilage microorganisms belonging to the genera *Pectinatus* and *Megasphaera.* Species specific AE labelled DNA probes were developed complementary to 16S r-RNA of each bacterium and a single genus specific probe was developed for detection of all three species of genus *Pectinatus.* The ATP bioluminescence method is routinely used in most of the breweries (Ehrenfeld *et al*., 1996; Franken *et al*., 2000). The luminometer is the basic instrument required for the ATP bioluminescence method and is therefore readily available in both medium size and large breweries. An application of a luminometer based method for detection of brewery contaminants using a highly sensitive chemiluminescence based method assay could be an easy and effective approach.

CHAPTER: 3 MATERIALS AND METHODS

CHAPTER 3 MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

Strains of beer spoilage bacteria and other related bacteria were obtained from DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany), ATCC (American Type Culture Collection), VTT (Valtion Teknillinen Tutkimuskeskus Culture Collection, Finland), Heineken Brewery, Netherlands and ICBD (International Centre for Brewing and Distilling, School of Life Sciences, Heriot Watt University, Edinburgh, UK). The species and the strains, specific media and incubation conditions used in this study are shown in Table 3.1.

All *Pectinatus* and *Megasphaera* strains were maintained on PYF medium (peptone-yeast extract- fructose, pH 7.0; medium 41, VTT Culture Collection, Finland). All *Lactobacillus* and *Pediococcus* strains were maintained on MRS (de Man, Rogosa, Sharpe media; de Man *et al*., 1962) agar and broth media (Oxoid, UK, medium CM0361 and CM0359 respectively) supplemented with 1 % sucrose. *Zymomonas mobilis* and *Micrococcus kristinae* were maintained on UBA (Universal beer agar; Kozulis *et al*., 1968; CM0651, Oxoid, UK) by incubating aerobically at 37 ºC for 4 days. *E. coli* (ICBD culture collection strain) was maintained on LB agar (Luria Bertani agar; Bertani, 1952; CM1021, Oxoid, UK). Freeze dried cultures were revived in 5 ml of specified broth for 12 hours at 30 ºC and then streaked on LB agar plates and incubated at 30ºC for 4 days.

For long term storage, 1 ml of actively growing bacterial culture was frozen in a 2 ml cryotube (Fisher- Thermo Scientific) in duplicate using liquid nitrogen and stored at -70 ºC, using 10-15 % sterile glycerol as a cryoprotectant. For *Pectinatus* and *Megasphaera*, 5-10 % anhydrous DMSO was used as the cryoprotectant (Suihko and Haikara, 1990).

For incubation purposes, the pre-reduction of autoclaved PYF and MRS broth media was carried out in aliquots of 10 ml by purging with an anaerobic gas mixture N_2:H_2:CO_2 (80:10:10) using a Don Whitley Mac 500 anaerobic cabinet followed by incubation of media in anaerobic conditions under an atmosphere of N_2:H_2:CO_2 (80:10:10) overnight at 30 ºC.

TABLE 3.1 Bacterial species and strains used for the study

Bacteria species	Culture collection strains	Media used	Incubation
Pectinatus cerevisiiphilus	ATCC 29359^, DSM 20467	PYF	Anaerobic at 30 ºC
Pectinatus frisingensis	VTT E 79100^, DSM 6306^	PYF	Anaerobic at 30 ºC
Pectinatus haikarae	VTT E 88330^, DSM 16980	PYF	Anaerobic at 30 ºC
Megasphaera cerevisiae	ATTC 43254, DSM 20461	PYF	Anaerobic at 30 ºC
Megasphaera sueciensis	DSM 17042	PYF	Anaerobic at 30 ºC
Megasphaera paucivorans	DSM 16981	PYF	Anaerobic at 30 ºC
Lactobacillus brevis	ICBD culture collection strain*	MRS+ 1% Sucrose	Anaerobic at 30 ºC
Lactobacillus casei	ICBD culture collection strain*	MRS+ 1% Sucrose	Anaerobic at 30 ºC
Lactobacillus lindneri	ICBD culture collection strain*	MRS+ 1% Sucrose	Anaerobic at 30 ºC
Lactobacillis paracollinodes	ICBD culture collection strain*	MRS+ 1% Sucrose	Anaerobic at 30 ºC
Lactobacillus plantarum	ICBD culture collection strain*	MRS+ 1% Sucrose	Anaerobic at 30 ºC
Lactobacillus coryniformis	ICBD culture collection strain*	MRS+ 1% Sucrose	Anaerobic at 30 ºC
Pediococcus damnosus	ICBD culture collection strain*	MRS+ 1% Sucrose	Anaerobic at 30 ºC
Pediococcus inopinatus	ICBD culture collection strain*	MRS+ 1% Sucrose	Anaerobic at 30 ºC
Pediococcus pentosaceus	ICBD culture collection strain*	MRS+ 1% Sucrose	Anaerobic at 30 ºC
Zymomonas mobilis	ICBD culture collection strain*	UBA agar	Aerobic at 37 ºC
Micrococcus kristinae	ICBD culture collection strain*	UBA agar	Aerobic at 37 ºC
Escherichia. coli	ICBD culture collection strain*	LB agar	Aerobic at 30 ºC

* Culture collection strain from the International Centre for Brewing and Distilling (ICBD), School of Life Sciences, Heriot Watt University, Edinburgh, UK; ^ Strains obtained from Heineken Brewery, Netherlands. PYF (peptone-yeast extract- fructose; medium 41, VTT Culture Collection, Finland), MRS medium (Oxoid, UK, medium CM0361), UBA agar (Universal beer agar; CM0651, Oxoid, UK), LB agar (Luria Bertani agar;CM1021, Oxoid, UK).

PYF, MRS and UBA agar were stored in the anaerobic cabinet for 12 hours before use to ensure anaerobic conditions. Working cultures of anaerobic bacteria were obtained by inoculating 10 μL of pure culture on specified agar plates and incubating in the anaerobic cabinet for 4 days at 30 ºC. A single colony was picked and inoculated into 10 ml of specified broth and incubated as described above.

3.2 Brewery samples

Based upon information on occurrence and survival sites of *Pectinatus* and *Megasphaera* in brewery environments (Back *et al*., 2005; Lee *et al*., 1980; Motoyama, 2003), all the sampling points were selected from the fermentation area, conditioning tanks and packaging sites, where anaerobic conditions could prevail or the sites are prone to biofilm formation. A schematic diagram of sample points is shown in Figure 3.1. All the samples were taken in the form of sterile swabs, rinse liquor, beer samples or yeast slurry.

The pre-reduction of autoclaved (MRS medium+ 1 % fructose) medium in aliquots of 62.5 ml was carried out by purging with an anaerobic gas mixture N_2:H_2:CO_2 (80:10:10) using a Don Whitley Mac 500 anaerobic cabinet followed by incubation of media in anaerobic conditions under an atmosphere of N_2:H_2:CO_2 (80:10:10) overnight at 30 ºC. Pasteurised commercial lager (4% ABV) was degassed in sterile containers by heating at 60 ºC with vigorous shaking and reduced by purging with anaerobic gas mixture N_2:H_2:CO_2 (80:10:10) followed by incubation of media in anaerobic conditions under an atmosphere of N_2:H_2:CO_2 (80:10:10) overnight at 30 ºC.

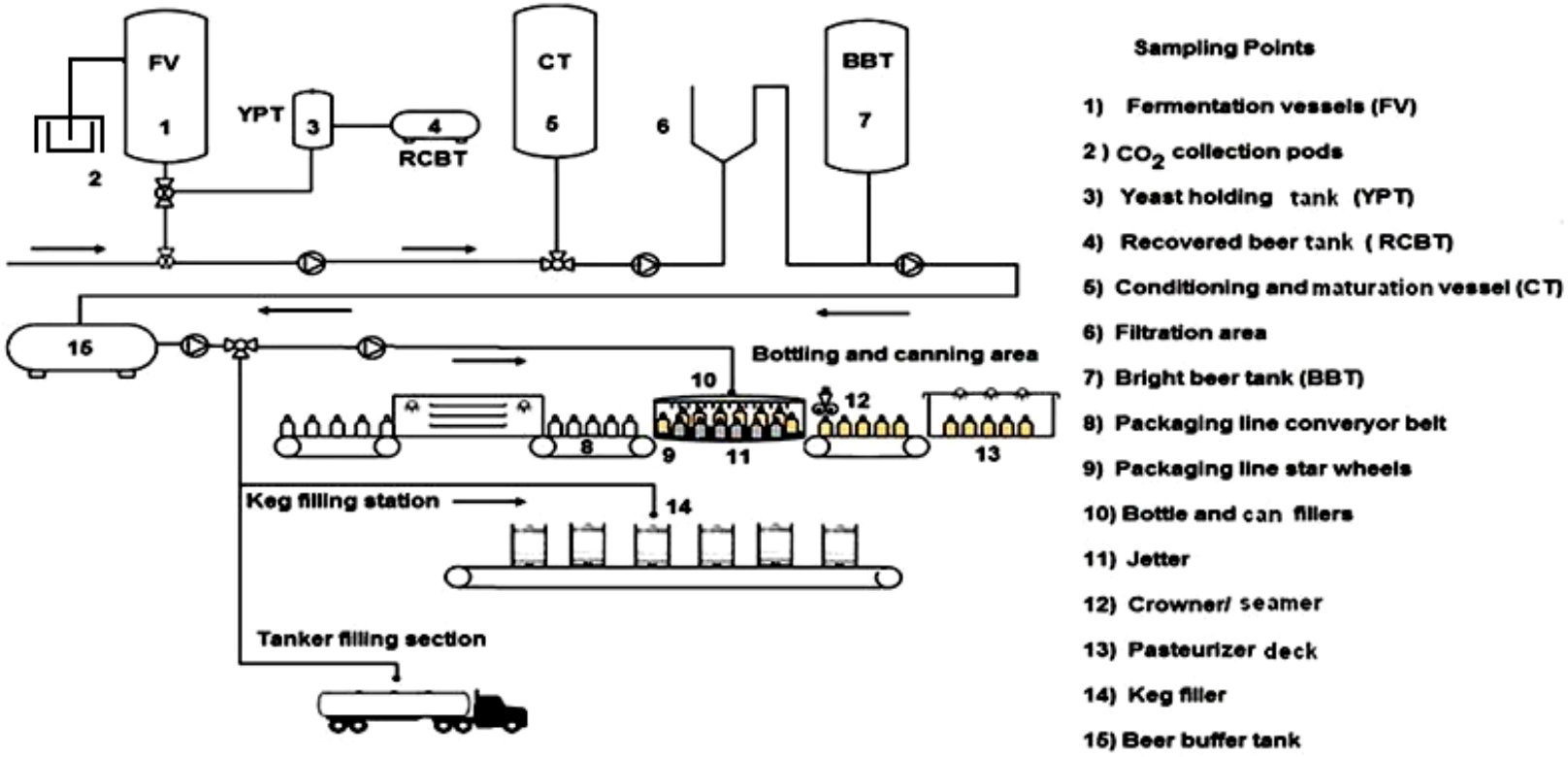


Figure 3.1 Schematic diagram of sampling points used during the project.

3.2.1 Swab samples

Swab samples were taken mainly from beer bottling lines and canning lines specifically from equipment and sites which come into direct contact with packaging materials or finished products. Swabs from bottle conveyor belts, in-feed and outlet star wheels, Jetters, Crowner and filler tubes were taken. Swabs were taken using sterile swabs and immediately inoculated into pre-reduced mixture of 250 ml volume of 25 % MRS broth + 1% fructose and 75 % pasteurised beer (4% ABV). Bottles were sealed with parafilm foil and maintained under anaerobic conditions using an anaerogen kit (Merck) in an anaerobic jar (Merck) at room temperature and transferred to an anaerobic chamber within 12 hours for further incubation.

3.2.2 Beer and rinse samples

Rinse samples mainly included samples from fillers and wash liquid from fermentation CO_2 collecting pods. Beer samples were selected from fermentation tanks, yeast holding tanks, bright beer tanks and beer buffer tanks. For rinse liquor and direct beer samples, liquid was directly poured into a sterile 250 ml bottle containing 62.5 ml pre-reduced MRS + 1% fructose medium. Bottles were sealed with parafilm foil and maintained under anaerobic conditions using an anaerogen kit (Merck) in an anaerobic jar (Merck) at room temperature and transferred to an anaerobic chamber within 12 hours for further incubation.

3.2.3 Yeast containing samples

For samples containing brewing yeast cells, 50 ppm cyclohexamide (to inhibit growth of yeast) was used to suppress the growth of yeast (Lee, 1994; Juvonen *et al.,* 2008). Samples were treated in a similar way as beer samples except, after incubation 100 μL of sample was inoculated on MRS agar + 1% fructose + 50 ppm cyclohexamide and incubated anaerobically for 4 days at 30 ºC.

3.3 Sample enrichment and cell harvesting

All samples were incubated at 30 °C for 14 days prior to DNA extraction. All enriched samples were centrifuged at 12000 rpm for 5 min to concentrate cells. A 500 µL aliquot of concentrated cell suspension was transferred to a 1.5 ml tube and washed 2-3 times with sterile deionised water with centrifugation at 12000 rpm for 1 min to recover the cell pellet. The final pellet obtained was frozen in liquid nitrogen and stored at -70 °C before being used for DNA isolation.

For some of the samples 100 µL of concentrated cell suspension in sterile deionised water was inoculated onto MRS agar + 1% fructose and incubated for 4 days under anaerobic conditions at 30 °C and DNA was extracted from representative colonies picked up and resuspended aseptically into 500 µL sterile deionised water. Following centrifugation, the pellet was frozen in liquid nitrogen and stored at -70 °C till further use.

3.4 Genomic DNA extraction and quantification

3.4.1 Genomic DNA extraction

DNA extraction was carried out using a Qiagen /Gentra- Puregene® kit according to the manufacturer's instructions. The kit is based on enzymatic- detergent lysis of bacterial cells, followed by degradation of RNAses and precipitation of cellular proteins. DNA was recovered using isopropanol precipitation and washed with 70 % ethanol.

The frozen or fresh cell pellet was suspended in cell suspension solution and 1.5 µL of lytic enzyme solution was added. The suspension was mixed by repeated pipetting. The tube was incubated at 37 °C for 1 hour to digest cell walls. After incubation the sample was centrifuged at 12000 rpm for 1 min and the supernatant was discarded and 300 µL of cell lysis solution was added to the cell pellet. The cell pellet was completely suspended in the lysis solution by pipetting and further incubated at 80 °C for 5 min to complete cell lysis. The sample was cooled on ice briefly for 1-2 min, 1.5 µL of RNAse-A solution was added to the sample and mixed by repeated pipetting and incubated at 37 °C for 1 hour. After incubation, the sample was cooled to room temperature and 100 µL of protein precipitation solution was added to precipitate cellular proteins. The suspension was mixed by vortexing and incubated on ice for 5 minutes. The sample was centrifuged at 12000 rpm for 3 minutes and the supernatant was transferred to a 1.5 ml microfuge tube containing 300 µL of 100 % isopropanol and mixed by inverting several times. The sample was then centrifuged at 12000 rpm, supernatant was carefully removed and 300 µL of 70 % (v/v) ethanol was added to the DNA pellet which was washed by gently inverting the tube several times. The sample was again centrifuged at 12000 rpm for 1 min and the tube was inverted on absorbent cloth and allowed to air dry for 15 min to remove traces of ethanol. Later, 50 µL of DNA hydration solution was added to the pellet and incubated at 65 °C for 1 hour. Alternatively samples were left at room temperature overnight to rehydrate DNA. The DNA isolates were stored at -20 °C.

3.4.2 DNA Quantification

DNA quantification was carried out using a UV spectrophotometer. Two µL of isolated DNA was added to 498 µL of TE buffer, pH 8.0 and absorbance was measured at 260 and 280 nm. DNA was quantified by using relationship the of 1 absorbance unit at 260 nm = 50 µg/ml of double stranded DNA. Absorbance at 280 nm was monitored to ensure the quality of DNA.

3.5 Multiplex PCR

PCR reactions were set up in four reaction formats for each of the three *Pectinatus* species (*Pectinatus* multiplex), three *Megasphaera* species (*Megasphaera* multiplex), six main beer spoilage *Lactobacillus* species (*Lactobacillus* multiplex) and three *Pediococcus* species as previously described by Asano *et al.* (2008) and Ijima *et al.*(2008). Certain modifications were made in the multiplex PCR method to ensure specificity and reactivity in order to overcome false positive or false negative results.

All primers were based on rRNA gene sequences and in some species the internal transcribed spacer (ITS) region. The details of primer sequences, target DNA and predicted product sizes are shown in Tables 3.2 and 3.3. All primers were purchased from Eurofins MWG Operon (UK). Stock solution of primers was carried out according to the manufacturer's instructions (to obtain a concentration of 100pmol/µL) using sterile deionised water and they were then stored at -20 °C.

3.5.1 *Pectinatus* multiplex

The *Pectinatus* multiplex was performed to detect three common beer spoilage *Pectinatus* species. *P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae* were detected in a single PCR reaction using an equimolar amount of primers 1 µL of (100pmol/µL). *P. frisingensis* gave two predicted amplified products with the pair of primers 16F-F and IF-R as forward primer 16F-F has two complementary sites resulting in production of two amplified products of 701 and 883 bp (Motoyama *et al.*, 2000).

3.5.2 *Megasphaera* multiplex

Megasphaera multiplex was performed to detect three beer spoilage *Megasphaera* species. In *Megasphaera* multiplex two pairs of primers were used; a single pair of primers was used to detect *M. cerevisiae*, whereas *M. paucivorans* and *M. sueciensis* were detected by a single pair of primers Msp-f and Msp-r, as these two species show 99 % similarity in their 16S rRNA gene sequence (Juvonen and Suihko, 2006). All primers were used in equimolar amount 1 µL of (100 pmol/ µL).

3.5.3 *Lactobacillus* multiplex

Lactobacillus multiplex PCR was performed to detect six *Lactobacillus* species as shown in Table 3.3. *Lactobacillus lindneri* was detected by species specific forward and reverse primers, LLITSF8 and LL23SR12, respectively. All other *Lactobacillus* species were detected using a species specific forward primer but primer UNP1 was shared as a common reverse primer by *L. brevis*, *L. casei*, *L. plantarum*, *L. corynformis* and *L. paracollinoides*. The *Lactobacillus* multiplex PCR was carried out using the same concentrations (LBP2-60 pmol, L74P1, LCP11, LOP4- 40 pmol, LLITSF8, LL23SR12- 80 pmol, LPP7= 120 pmol and UNP1-100 pmol) of primers as described by Asano *et al*. (2008).

Predicted size (bp) of amplified PCR products for *L. brevis*, *L paracollinoides* and *L. lindneri* were 861, 854 and 851 bp respectively. As these products were difficult to resolve using 2 % agarose gel electrophoresis, simplex PCR was performed to detect individual samples at species level. The simplex PCR was performed in a similar way as described in section 3.5.5 except 10 pmol of each of species specific forward and reverse primer were used (as shown in Table 3.3)

3.5.4 *Pediococcus* Multiplex

In the original protocol described by Ijima *et al*. (2008), *Megasphaera* and *Pediococcus* multiplex were performed in a single reaction as a cocci multiplex (Beer spoilage Cocci multiplex), but *Pediococcus* multiplex was performed as a separate reaction in the present study. Equimolar concentrations 1µl of (100 pmol/µl) of each primer were used. *P. damnosus* and *P. inopinatus* were detected using the same pair of primers (PIDF-1 and PID8R as forward and reverse primer respectively).

Table: 3.2. List of primers used for detection of *Pectinatus* and *Megasphaera* (Iijima *et al.*, 2008)

Method	Primer*	Direction	Primer sequence (5' to 3')	Target species	Target DNA	Product size (bp)
	16C-F	Forward	CGTATGCAGAGATGCATATT	*P. cerevisiiphilus*	16S- rDNA	621
Pectinatus	IC-R	Reverse	CACTCTTACAAAGTATCTAC	*P. cerevisiiphilus*	ITS region	
Multiplex	16F-F	Forward	CGTATCCAGAGATGGATATT	*P. frisingensis*	16S-rDNA	701, 883
	IF-R	Reverse	CCATCCTCTTGAAAATCTC	*P. frisingensis*	ITS region	
	Phf1	Forward	AATACCGAATGTTGTAAGAG	*P. haikarae*	16S-rDNA	508
	Phr2	Reverse	CTCTCCTGCACTCAAGACAT	*P. haikarae*	16S-rDNA	
Megasphaera	mc-f4	Forward	ACCGAATACGATCTAAAG	*M. cerevisiae*	16S-rDNA	452
Multiplex	mc-rf	Reverse	TTAAGACCGACTTACCGA	*M. cerevisiae*	16S-rDNA	
	Msp-f	Forward	TATGGCCAATACCCATAGAT	*M. sueciensis &*	16S-rDNA	155
	Msp-r	Forward	CACTTTTAAGACAGACTTGA	*M. paucivorans*	16S-rDNA	

*- All *Pectinatus* and *Megasphaera* multiplex primers were used in equimolar concentration (100pmol in 50 µl reaction volume)

Table: 3.3. List of primers used for detection *of Lactobacillus and Pediococcus* (Iijima *et al.*, 2008)

Method	Primer	Direction	Primer sequence (5' to 3')	Target species	Target DNA	Product size (bp)
	LBP2	Forward	CTGATTTCAACAATGAAGC	*L. brevis*	16S-rDNA	861
Lactobacillus	L74P1	Forward	GGATTTTAACATCGGATGAG	*L. paracollinoides*	16S-rDNA	854
Multiplex	LCP11	Forward	GAACCGCATGGTTCTTGGC	*L. casei*	16S-rDNA	729
	LOP4	Forward	GGGACTAGAGTAACTGTTAGTCC	*L. corynformis*	16S-rDNA	453
	LPP7	Forward	GTTGTTAAAGAAGAACTTATC	*L. plantarum*	16S-rDNA	490
	LLITSF8	Forward	AACTTACACCGATCAAAATC	*L. lindneri*	ITS region	850
	LL23SR12	Reverse	CTTAACCTTGCATGCAACT	*L. lindneri*	16S-rDNA	-----
	UNP1	Reverse	CCGTCAATTCCTTTGAGTTT	*Lactobacillus spp. (consensus primer)*	23S-rDNA	*
Pediococcus	PIDF1	Forward	ACCGAATACGATCTAAAG	*Ped.damnosus*	16S-rDNA	566^
Multiplex	PID8R	Reverse	TTAAGACCGACTTACCGA	*Ped. inopinatus*	16S-rDNA	-----
	PCLAF3	Forward	TGTGAGAGTAACTGCTCATG	*Ped. claussenii*	16S-rDNA	462
	PCLAR3	Reverse	ACGCCTAATCTCTTTGGTTA	*Ped. claussenii*	16S-rDNA	

* Primer- UNP1 is shared as a common reverse primer by all 5 *Lactobacillus* species except *L. lindneri*

^ *P. damnosus* and *P. inopinatus* shared a common forward and reverse primer: Ijima *et al.* 2008.

Amount of primers used in 50 µl reaction volume (LBP2-60 pmol, L74P1, LCP11, LOP4- 40 pmol, LLITSF8, LL23SR12- 80 pmol, LPP7= 120 pmol and UNP1= 100 pmol) (Asano *et al*., 2008)

All *Pediococcus* multiplex PCR primers were used at equimolar concentration (100pmol in 50 µl reaction volume)

3.5.5 PCR conditions and gel electrophoresis

For each reaction mixture 0.5µL (2.5 units) of BIOTAQ ™ DNA Polymerase (BIOLINE) was used. Standard reaction buffer containing a final concentration of 0.8 mM $(NH_4)_2SO_4$, 3.5 mM Tris-HCL, 1.5 mM $MgCl_2$ and 0.2 mM of each of the four dNTPs was used. For *Pectinatus*, *Megasphaera* and *Pediococcus* multiplexes 1µL of each primer (100pmol/µL) was used. For *Lactobacillus* multiplex primer concentrations were as previously described by Asano *et al*. (2008). 1µL (500-700 ng/µL) of extracted DNA solution was used as a template and the final volume of reaction mixture was made to 50 µL using sterile deionised water. PCR reactions were performed using BIORAD and Applied Biosystem thermal cyclers. A positive control was set up by using 1 µL DNA template of *P. frisingensis, M. cerevisiae* and *L. brevis* for *Pectinatus, Megasphaera and Lactobacillus* multiplex PCR respectively. A negative control was included using a reaction mixture as described above except no DNA template was used.

The PCR amplification was carried out with an initial denaturation for 4 minutes at 95 °C followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 56 °C for 30 seconds and primer extension at 72 °C for 1 minute. Final primer extension was carried out for 4 minutes at 72 °C followed by an end hold at 4 °C. PCR products were stored at 5-6 °C before analysis by gel electrophoresis using 2 % agarose gels in TAE buffer (0.04 M Tris-acetate, 0.001M EDTA, pH 8.0) containing ethidium bromide (0.2µg/ml) for DNA staining. A 5 µl aliquot of PCR product was used for analysis and a 100 bp ladder (Hyper ladder IV- BIOLINE) was used as molecular size marker.

For *Lactobacillus* multiplex, certain similar sized amplified fragments were confirmed using simplex PCR as described above, except 1 µL (10 pmol/ µL) each of species specific primer was used.

3.5.6 Determination of specificity of primers

The specificity of all the primers to amplify the predicted DNA fragment was detected in multiplex format. The species specific pair of primers was tested for production of any unpredicted amplification products by using 1 µL (500-700 ng/µl) of DNA isolated from all the bacterial cultures shown in Table 3.1. PCR reactions were carried out as described in section 3.5.5.

3.5.7 Determination of sensitivity of primers

Determination of sensitivity of all the multiplex PCR was assessed to ensure minimum detection level of multiplex PCR methodology applied in this study. The concentration of target bacterial species DNA was determined by UV spectrophotometer by measuring absorbance at 260 and 280 nm as described in section 3.4.2. The purified bacterial DNA (1ng) was diluted serially diluted and used for PCR regimes as described in section 3.5.5. The amplified products were separated on 2 % agarose gel and positive results were evaluated based on visible bands observed. The sensitivity of simplex PCR with a species specific pair of primers was also determined using the same procedure as described above except 10 pmol of each of the primers was used.

3.6 Isolation of *Pectinatus* and *Megasphaera*

The samples found positive for either *Pectinatus* or *Megasphaera* were preserved for further studies. 1 ml centrifuged aliquots were frozen in 2 ml cryotubes (Fisher-Thermo Scientific) using liquid nitrogen and stored at -70 º C. Anhydrous DMSO (5-10 %) was used as a cryoprotectant.

Frozen *Pectinatus* and *Megasphaera* positive samples (50-100 µl) were revived in 5 ml of SMMP broth incubated anaerobically at 30 ºC for 4 days, later 100 µL of revived sample in SMMP broth was inoculated onto SMMP agar medium and incubated at 30 ºC for 4-7 days. Between 5 and 10 representative colonies of each of *Pectinatus* and *Megasphaera* isolates were picked from each individual plate and further sub- cultured on SMMP agar. The plates were sub-cultured every 2 weeks.

3.7 Characterisation of *Pectinatus* and *Megasphaera* isolates

3.7.1 General characterisation

The brewery isolates of *Pectinatus* and *Megasphaera* were Gram stained and the motility of bacterial cultures was checked using wet mount and 100X magnification on a phase contrast microscope. Photographs were captured using a Zeiss monochrome camera. The colony morphology of bacterial isolates was checked on PYF, MRS and SMMP medium after anaerobic incubation at 30 ºC for 7 days.

3.7.2 Scanning electron microscopy

Bacterial isolates were grown anaerobically in 5 ml of PYF broth at 30 ºC for 12 to 18 hours. A 100 µL aliquot of cell culture was suspended in a 1.5 ml tube containing 400 µL of pre-reduced PYF media and vortexed briefly to ensure uniform suspension. A 500 µL aliquot of bacterial suspension was added to a single well of an eight well strip plate containing thin circular glass cover slips and incubated anaerobically at 30 ºC for 12-18 hours.

After incubation, bacterial medium was removed by pipetting without disturbing the glass cover slip and the cover slip was washed with PBS buffer (pH 7.2) 3 times, each time for 5 min. The cells were fixed on the cover slip using 1ml of 2.5 % glutaraldehyde solution in PBS buffer (pH 7.2). The incubation was carried out for 2 hours and glutaraldehyde solution was then removed by pipetting and the glass cover slip was washed with PBS buffer (pH 7.2) 3 times, each time for 5 minutes. Dehydration of bacterial samples was carried out using 1 ml of different ethanol concentrations. The samples were treated with 30 %, 50%, 70%, 80 %, 90 % and 100 % ethanol for 10 min each and cover slips were stored overnight in excess of 100 % ethanol at 4 ºC. Critical point drying was carried out using a critical point dryer (Peak Scientific Ltd., UK) for roughly 3 hours and cover slips were mounted on aluminium stubs using two sided sticky tape and kept desiccated using silica beads. The samples were sputtered with gold particles and visualised under the electron microscope (Quanta™ 3D FEG-FEL) and images were captured in the Electron Microscopy facility within the School of Engineering and Physical Sciences, Heriot Watt University, Edinburgh.

3.8 Biochemical characterisation

The *Pectinatus* and *Megasphaera* isolates were checked for catalase, oxidase activity and utilisation of different carbohydrate substrates and also for production of acids from these substrates. Growth was monitored at different pH and temperatures and the ability to spoil different beers (0-6.6 % ABV) was also evaluated. Antibiotic susceptibility of these microorganisms was also determined and the results were compared with the corresponding culture collection strains.

3.8.1 Carbohydrate utilisation profiling

The sugar utilisation ability of *Pectinatus* was monitored using API-50CHL (*BioMe´rieux*, UK).

For *Pectinatus* isolates, bacterial colonies were picked up from PYF agar and suspended into 10 ml API 50 CHL medium (peptone 10 g, yeast extract 5 g, tween 80 1 ml, dipotassium phosphate 2 g, sodium acetate 5 g, diammonium citrate 2 g, magnesium sulfate 0.20 g, manganese sulfate 0.05 g, bromcresol purple 0.17 g, in deionised water 1000 ml, pH 6.7-7.1) to obtain a uniform turbid suspension (0.45 absorbance at 600 nm). The wells were filled with bacterial suspension to the line mark and covered with mineral oil. The API-50 CHL set up was incubated anaerobically at 30 ºC for 48 hours and readings were taken at 24 hour intervals. The positive results were evaluated based on appearance of visible turbidity and colour change in media.

3.8.2 Determination of bacterial growth at different pH range

Bacterial isolates were grown overnight in PYF medium anaerobically at 30 ºC and 500 µl was inoculated into a series of 10 ml aliquots of PYF medium in duplicate, with the pH of the PYF medium adjusted from 3 to 10 at intervals of 0.5 unit. The inoculated medium was incubated anaerobically at 30 ºC for 4 days and positive results were identified based on visible growth in PYF medium.

3.8.3 Determination of bacterial growth at different temperatures

The bacterial isolates were streaked on PYF agar and incubated under anaerobic conditions using an anaerogen kit (Merck) at different temperatures (4, 15, 30, 37, 45 ºC). The results were obtained after 7 days of incubation based on formation of visible colonies.

3.8.4 Antibiotic susceptibility

The antibiotic susceptibility of bacterial strains was evaluated using the MASTRING MID8 ANAEROBE ID RING through an agar diffusion method. The bacterial culture was grown in 10 ml of PYF broth at 30 ºC for 12 to 18 hours. The turbid suspension (approximately 1x 10^8 cells/ ml) was uniformly spread plated on PYF agar plates using a sterile swab and a MASTRING MID8 ANAEROBE ID RING was placed on the inoculated media and the plates were incubated in anaerobic conditions for 7 days at 30 ºC. The plates were checked for clear zone formation every day.

3.8.5 Determination of beer spoilage ability

Commercial lagers with different % ethanol concentrations (0 %, 2 %, 4 % and 6.6 % ABV) were obtained (Table 3.4) to evaluate beer spoilage ability of *Pectinatus* and *Megasphaera* isolates. The ABV values were noted from the beer labelling. The pH values of the beers were determined by using a pH meter. A 500 µL aliquot of overnight grown bacterial culture was added to 100 ml degassed beer and incubated anaerobically at 30 ºC for 14 days. The bacterial growth was monitored using a spectrophotometer by measuring absorbance at 600 nm at intervals of 12 hours.

Table: 3.4 Properties of beer used for the study

Beer [1]	% ABV[2]	Hop bitterness[3] (IBU)	pH[4]
Dutch Lager	0	15	3.9
English Lager	2	18	3.8
Scottish Lager	4	24	4.1
Italian Lager	6.6	28	4.1

1- All lagers were obtained from a local supermarkets

2- % ABV values were noted from the beer labelling

3- Hop bitterness (iso α- acids (mg/L) was determined using ASBC method (1992).

4- pH values were determined for degassed beer at room temperature

3.8.5.1 Determination of beer bitterness

The beer bitterness was determined using the standard ASBC method (ASBC, 1992). In a 50 ml centrifuge tube , 10 ml of beer was taken, 1ml of 3M HCl and 20ml of 2,2,4-trimethylpentane was added and the tube was shaken vigorously for 15 minutes. If two liquid phases were not separated after shaking the sample was centrifuged at 6000 rpm for 2 min and the clear upper (2,2,4-trimethylpentane) layer was transferred to a glass cuvette. The absorbance was measured at 275 nm in a UV spectrophotometer (Thermo Scientific GENESYS-6) against 2, 2, 4-trimethylpentane. Readings were taken in duplicate and the average absorbance reading was multiplied by 50 to obtain concentration of iso-α acid (mg/L) typically expressed in International Bitterness Units (IBU).

3.8.5.2 Determination of concentration of acetic acid and propionic acid

Two ml of samples were taken and filtered through 0.45 µm and stored at -20 ºC. Acetic acid and propionic acids were analysed by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC). 1:10 (v/v) samples were first passed through Solid Phase Extraction using a Varian Bond SAX column. The solid phase extraction column was treated with 3ml of deionised water and eluted with 1.5ml of 1M hydrochloric acid**.** The prepared sample was injected into the HPLC system, composed of Waters 484 tunable wavelength detector Jasco series II and III pumps, Spectra systems AS1000 auto sampler, Jasco 880-50 degasser and CSW 32 data handling (HP3365). Compounds were separated using a reverse phase column Phenomenex, Synergy 4u hydro-RP 80A, 250 x 4.6mm. The samples were eluted using eluent A; 20mM potassium phosphate (monobasic, anhydrous), pH 2.5 with 5 M HCl and eluent B: Water: Acetonitrile (1:1). Gradient of eluent B was increased from 0 % to 100 and decreased to 0 % over the period of 68 mins and flow rate of 0.5 ml/ min was maintained. For calibration, acetic acid and propionic acid were obtained from Sigma (HPLC grade).

3.9 Genetic characterisation

3.9.1 Amplification of 16S ribosomal RNA gene

The bacterial isolates were grown on PYF agar for 4 days at 30 ºC and a single colony was picked and suspended in 20µl sterile deionised water. The cells were lysed by heating at 95 ºC for 10 min. After heat lysis of cells, the sample was snap-cooled on

ice for 3 min. The sample was briefly centrifuged at 12000 rpm for 1 min to pellet cell debris and 1 µl of supernatant was used as crude DNA extract. The PCR was carried as described in section 3.5.5, with species specific primers (Table 3.2) or by using bacterial universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 685r (5'-TCTACGCATTTCACCGCTAC-3') as forward and reverse primers respectively. 10 pmol amounts of both primers were used for PCR amplification. The successful amplification was confirmed by running the PCR products on a 2 % agarose as described in section 3.5.5.

The PCR products were purified using a QIAquick spin column (Qiagen) to remove dNTPS, unreacted primers and other impurities according to the manufacturer's instruction. In brief, the PCR amplified samples were bound to silica based membrane in the spin column and impurities were removed by application of high salt buffers. The final elution was carried out using 50 µl of TE buffer (pH-8.0). The concentration of PCR amplified product was determined using a UV spectrophotometer as described in section 3.4.2 and the product diluted to a concentration of 20 ng/µl and stored at 4 ^{o}C till further use.

3.9.2 Partial 16S ribosomal gene sequencing

The samples were sequenced in both forward and reverse directions, to minimise the PCR artefacts, ambiguities and base-calling errors. In 0.2 ml PCR tubes, 5 µL of PCR amplified product (20 ng/µl) was mixed with 1 µl either of forward or reverse primer (3.2 pmol/µl). The sequencing was performed on an automated ABI Prism 3730 Genetic Analyzer using ABI Big Dye v3.1 Terminator sequencing chemistry (Applied Biosystems, Foster City, CA) in the GenePool Sanger sequencing facility at University of Edinburgh (http://genepool.bio.ed.ac.uk).

3.9.3 Analysis of 16S ribosomal gene sequence

Sequencing Chromatograms were visualised using Finch TV version1.4. The forward and reverse sequences were aligned in Clustal-W and both the sequences were merged using Emboss Merger software (http://emboss.bioinformatics.nl/cgi-bin/emboss/help/merger) and flanking sequences were removed. The merged sequences were used for nucleotide BLAST searches to determine the phylogenetic similarities with existing strains of bacteria (Johnsons *et al*., 2008).

3.10 Hybridisation Protection Assay

3.10.1 Preparation of working cultures

Working cultures were obtained by inoculating 10 µL of pure culture onto PYF agar plates and incubating in anaerobic conditions under an atmosphere of N_2:H_2:CO_2 (80:10:10) for 4 days at 30 ºC. A single colony was picked and inoculated into 10 ml of PYF broth then further incubated for 4 days as described above.

3.10.2 Bacterial RNA stabilisation

A 500 µl aliquot of the overnight culture containing approximately 10^8 cells/ ml was mixed with 1000 µl of bacterial RNA stabilization solution (Qiagen); the suspension was vortexed briefly and incubated at room temperature for 10 minutes. The suspension was then centrifuged at 12000 rpm for 10 minutes and the supernatant was removed by pipetting. The pellets were quickly frozen in liquid nitrogen and stored at -70 ºC till further use.

3.10.3 Total cellular RNA isolation

The RNA extraction was carried out using an RNeasy® mini Kit (Qiagen) following the manufacturer's instructions. The RNA stabilized bacterial pellet was mixed with 200 µL of cell lysis buffer consisting of TE buffer (30 mM Tris, 1 mM EDTA, pH 8.0), lysozyme (15 mg/ml) and 20 µL Proteinase K (Qiagen). The pellet was suspended in lysis buffer by repeated pipetting and incubated at 37 ºC for 10 min with continuous shaking. 700 µL RLT buffer, containing β- mercaptoethanol was added to the cell lysate and vortexed for 20 sec followed by addition of 500 µL of 96 % ethanol. The suspension was vortexed briefly and 700 µL of suspension was added to an RNeasy mini spin column, placed in a 2 ml collection tube. The sample was centrifuged at 10000 rpm for 15 sec, the flow through was discarded and the procedure was repeated for the remaining suspension. RW1 reagent (700 µL), was added to the spin column and centrifuged at 10000 rpm for 15 sec and the flow-through was discarded with the collecting tube. The spin column was placed in new collecting tube and washed with 500 µL RPE buffer at 10000 rpm for 15 sec and then for 2 min; flow-through was discarded each time. Finally, RNA was eluted by addition of 50 µL deionised RNAse free water and centrifuging at 10000 rpm for 1 min. The RNA solution was divided into single use aliquots of 10 µL, frozen in liquid nitrogen and stored at -70 ºC till further use.

3.10.4 Total cellular RNA quantification

Total RNA quantification was carried using a UV spectrophotometer: 2 µL of isolated RNA was added to 498 µL of TE buffer (pH 8.0) and absorbance was measured at 260 and 280 nm using a SHIMADZU- UV-1650PC UV-VIS spectrophotometer. RNA was quantified by using the relation of 1 absorbance unit at 260 nm = 40 µg/ml of RNA.

3.10.5 Determination of quality of total cellular RNA

The quality or stability of total cellular RNA was observed using denaturing formaldehyde gel electrophoresis as follows: 3 µl of RNA solution was mixed with 6 µl of denaturing loading buffer [50 % (v/v) formamide, 16 % (v/v) formaldehyde, 10 % (v/v) of 10x MOPS buffer (200mM MOPS, 50mM sodium acetate, 10mM EDTA, pH-7), 0.1 µL ethidium bromide (10mg/ml) and 0.01 % of bromo-phenol blue], incubated for 10 minutes at 72 ºC to denature the RNA and snap-cooled on ice for 3 minutes. The RNA solution was then run on a denaturing 1 % agarose gel containing 10 % (v/v) of formaldehyde and 1x MOPS buffer (20mM MOPS, 5mM sodium acetate, 1mM EDTA, pH-7.0) and 1x MOPS was used as tank loading buffer. DEPC (0.1%) -treated sterile deionised water was utilized for preparation of all the reagents and RNasezap® (Ambion Biotech) was used for cleaning of work benches and electrophoresis apparatus to inhibit any RNase activity.

3.11 Selection and modification of DNA probes

Probes specific to 16S RNA sequence were selected from previous publication (Yasuhara, 2001) and three new probes were designed for the detection of *P. cerevisiiphilus*, *P. haikarae* and a common probe was designed for *Pectinatus* species. One species specific probe was designed for *M. cerevisiae* and a common DNA probe was designed for *M. paucivorans* and *M. sueceinsis*.

The new probes were designed using LNA™ probe designer software (www.exiqon.com). The probes were analysed for specificity using nucleotide BLAST search tool (http://blast.ncbi.nlm.nih.gov; Johnson *et al.,* 2008) and the probe-match tool (http://rdp.cme.msu.edu/probematch/search.jsp; Kim *et al*., 2009). The probes used in the present study are shown in Table 3.5. The oligonucleotide probes were obtained from MWG Eurofins, UK. Each probe was modified using two strategies; a

modification by inserting a C_5 amine linker arm at the 5′ end during synthesis and an internal modification by inserting amine-modified thymidine base.

Additionally all the probes labelled with DIG at the 3′ end were obtained from MWG Eurofins, UK as freeze dried pellets. The DIG labelled probes later were diluted to obtain a concentration of 50 pmol/ µL using sterile deionised water and stored at -70 ºC. The DIG labelled probes were used for RNA detection using RNA slot blots.

Table: 3.5 Sequences of oligonucleotide probes used for HPA assay and RNA slot blot analysis

Probe[d]	Oligonucleotide Sequence [a, b] (5'---3')	Target gene	Target bacteria	References
PC	GCA TCT CTG CAT*ACG TCA ATC AAT GTC	16S r-RNA gene	*P. cerevisiiphilus*	This study
PF[c]	AAG ATC CGC TTA ATG TT*C CGC CTG CG	16S r-RNA gene	*P. frisingensis*	Yasuhara *et al., 2001*
PH	CCT GCA CTC AAG ACA T*TC AGT TCG GA	16S r-RNA gene	*P. haikarae*	This study
PCFH	TTA CCG TCA CCA ACT AGC TAA T*CA GAC	16S r-RNA genes	*P. cerevisiiphilus* *P. frisingensis* *P. haikarae*	This study
MC	CAGGATATCTCTATCCCT*GGCACTCAA	16S r-RNA gene	*M. cerevisiae*	This study
MPS	ATCTCTGCCTCGTT*CAATCAATGTCA	16S r-RNA genes	*M. paucivorans* *M. sueceinsis*	This study

[a] -same sequence was used for 5' end amine modification internal thymidine base modification and 5' DIG labelling.

[b] -PCFH probe represents *Pectinatus* genus specific probe

[c] -The original sequence was extended by 6 bases at 3' end.

[d] -All 5' end labelled probes are denoted as (PC-1, PF-1, PH-1, PCFH-1, MC-1 and MPS-1) and all internally labelled AE probes are referred as (PC-2, PF-2, PH-2, PCFH-2, MC-2 and MPS-2) in the text.

*- Represents internal amine- modified thymidine base for covalent attachment of acridinium ester (internally labelled AE probes only)

3.11.1 5' end amine modification of DNA probes

The 5' end amines labelled probes were obtained from MWG Eurofins, UK. The DNA probes were synthesised according to standard solid-phase phosphoramidite chemistry. All the designed DNA probes were modified during synthesis by inserting a C_6 amine linker at the 5' end as described by Arnold *et al*., 2000. The scale of synthesis was 0.05 µM and probes were purified using reverse -phase HPLC.

3.11.2 Internal amine modification DNA probes

The internal modification of the DNA probes was carried out by inserting an amine modified thymidine base instead of a normal thymidine base in the oligonucleotide probe sequence during synthesis. The amine modified thymidine base was inserted at the point of a mismatch nucleotide or within two bases of a mismatch to increase the specificity of the acridinium ester labelled DNA probes.

3.11.3 Ethanol precipitation of DNA probes

Amine modified DNA probes were synthesised by MWG Eurofins and obtained in the form of freeze dried pellets, The DNA probes were purified using ethanol precipitation as follows: The DNA probe was dissolved in sterile deionised water to obtain a concentration of 5 µg/ µL. The DNA probe (10 nmol- approximately 80-90 µg) was precipitated by addition of $1/10^{th}$ volume of 3 M sodium acetate (pH-5.2), 2 fold volume of chilled 96 % ethanol and 2 µL (15mg/ml) of glycogen (Glycoblue™ - Invitrogen) followed by incubation at -20 ºC for 30 minutes. After incubation the DNA oligonucleotide probe was pelleted by centrifuging at 12000 rpm for 10 minutes and used directly for labeling reactions.

3.12 Labelling and purification of DNA probes

The succinimidyl derivative of the acridinium ester (9[[4-[3-[(2,5- dioxo-1-pyrrolidinyl) oxy] -3- oxopropyl] phenoxy] carbonyl] -10-methyl- acridinium trifluoromethane sulfonate) (Weeks *et al*., 1983) was obtained from Cambridge Biosciences; the working stock was obtained by dissolving AE in anhydrous DMSO to obtain a final concentration of 25mM. The working stock was prepared freshly for each use and used immediately after preparation. The main AE stock was stored desiccated using silica beads in an airtight container at -70 ºC.

3.12.1 DNA oligonucleotide labelling

The labelling was carried out as described by Arnold *et al*. (1993) and Mazumdar *et al*. (1998). Ten nmol of ethanol precipitated DNA probe in a 1.5 ml tube was used for labelling. The ethanol precipitated pellet was dissolved in 10 µl of solution mixture (3 µl deionised water, 1 µl 1M HEPES (pH 8.0), 4 µl anhydrous DMSO and 2 µl of 25 mM acridinium ester working stock in anhydrous DMSO). The DNA probe pellet was dissolved by vortexing and then briefly centrifuged for 10 sec to collect components at the bottom of the tube. The mixture was incubated at 37 ºC for 20-30 min and the content of the tubes were mixed by gentle shaking every 5 min. After incubation 3 µl of 25 mM acridinium ester working stock in anhydrous DMSO, 1.5 µl of deionised water and 0.5 µl of 1M HEPES buffer (pH-8.0) were added sequentially to the tube. The tube was vortexed for 10 sec and briefly centrifuged for 10 sec and incubated for a further 20-30 min at 37 ºC. The reaction was quenched using 5 µl of 0.125 mM lysine in 0.1 M HEPES (pH- 8.0) and incubated at 37 ºC for 10 min.

The AE labelled DNA probes were separated from unreacted acridinium ester using a DyeEx spin column (Qiagen) according to the manufacturer's instructions and further ethanol precipitated as follows: 20 µl of DNA probe from the spin column was mixed with 30 µL of 3M sodium acetate (pH 5.0), 245 µl of sterile deionised water and 5 µl of glycogen (Glycoblue- Invitrogen). The solution was mixed by vortexing. Absolute ethanol, (640 µl) was added to the tube and vortexed briefly to mix the contents. The tube was incubated on ice for 30 min and centrifuged at 13000 rpm for 10 min; the supernatant was discarded. The AE labelled DNA probe pellet was dissolved in 20 µl of 0.1 M sodium acetate (pH-5.0), 0.1 % SDS and stored at -20 ºC till further use.

3.12.2 Reverse phase HPLC Purification

The ultra purification of AE labelled DNA probe was carried out using reverse phase High Pressure Liquid Chromatography (RP-HPLC). A Vydac C_4 column (214TP1010**)**, (Western analytical, USA) was used for the purification using a binary buffer system as previously described by Arnold *et al.,* (1993); Nelson *et al*., (1995) and Mazumdar *et al.,* (1998). TEAA (triethyl ammonium acetate, pH- 7.0; 0.1 M) and acetonitrile were used for elution. The linear gradient of buffer was maintained and the concentration of acetonitrile was increased from 10 % to 40 % over the period to 30 min and a flow rate of 0.5 ml/ min was maintained. The elution was monitored by measuring absorbance at 260 nm using Waters 484 tunable absorbance detector (Waters, UK) and

desired fractions were collected and pooled together. The pooled fractions containing AE labelled DNA probe were ethanol precipitated as described in Section 3.11.3 and dissolved in 20 µl of 0.1 M sodium acetate (pH-5.0), 0.1 % SDS and stored at -70 ºC.

3.13 Determination of specific activity of AE probes

The specific activity of the AE labelled DNA probes was calculated as described by Nelson *et al*. (1995) and Nelson *et al*. (1996). In brief, chemiluminescence per µl of AE labelled DNA probe in the stock solution was measured by serially diluting the AE DNA stock solution and measuring chemiluminescence in a dual injector Luminometer (Berthold LB 9760). The percent contribution of DNA probe and AE molecule to absorbance at 260 nm was evaluated using extinction coefficient values of DNA oligonucleotide and AE molecule respectively and the specific activity of the DNA probe (RLUs/pmol) was calculated as described by Nelson *et al*. (1995).

3.13.1 Measurement of chemiluminescence

Serial dilutions of AE stock solution were prepared; 2 µl of AE solution was tenfold serial diluted in 0.1 M sodium acetate, 0.1 % SDS. 10 µl of each dilution was added to 190 µl of 5% Triton X-100 and mixed by vortexing. The chemiluminescence was measured for a total volume of 200 µl using a dual injector Luminometer (Berthold-LB 9760) by automatic injection of 200 µl of 0.4 M HNO_3, 0.1 % H_2O_2 then 200 µl of 1 M NaOH with a 1 second delay, followed by measurement of chemiluminescence for 5 seconds. The values were expressed in Relative Light Units (RLUs). The measurement was repeated three times for each dilution. The amount of chemiluminescence per µl of stock solution was calculated by taking average values for each dilution (ranging between 50,000-250,000 RLUs), with dilution factors being taken into consideration.

3.13.2 Determination of concentration of AE-labelled DNA probe

Two µl of DNA probe was diluted in 398 µl of TE buffer (pH-8.0) and absorbance was measured at 260 nm using a UV spectrophotometer. The percent contributions of DNA probe and acridinium ester molecules to absorbance at 260 nm were calculated by determining extinction coefficient values for the DNA probe and AE molecule (Nelson *et al*., 1995). The amount of DNA probe (pmol/ µl) was calculated. The specific activity (RLUs/pmol) of the probe was determined and stock solution was diluted in 0.1 M sodium acetate containing 0.1% SDS to give a concentration of 1 pmol/ µl. The 20 µl aliquots were prepared and stored at -70 ºC till further use.

3.14 Determination of differential hydrolysis (DH) kinetics of AE probes

Differential hydrolysis (DH) kinetics determine the rate of hydrolysis of hybridised and unhybridised DNA probe at a given pH and temperature (Nelson *et al*., 1993; Nelson *et al*., 1996). The differential hydrolysis reaction was performed using 1pmol of target RNA molecules and 0.1 pmol of AE DNA probe (Nelson *et al*., 1995).

In 30 µl of succinate buffer 1 (0.1 M lithium succinate (pH 5.2), 2 mM EDTA, 2mM EGTA, 10 % lithium dodecyl sulphate (w/v)), 1 pmol of target RNA and 0.1 pmol of AE DNA probe was added. A negative control was maintained in a similar way except target RNA was not added and a blank was maintained with no AE labelled probe. The reaction mixtures were incubated at 60 ºC for 45 minutes. The reaction mixtures were then diluted by addition of 270 µl of succinate buffer 1. Ten µl aliquots were prepared in luminometer tubes (Lumivials, Berthold) and differential hydrolysis was carried out by adding 100 µl of 0.125 M sodium tetraborate (pH 8.0), 5 % Triton X-100. The tubes were incubated at 60 ºC. Samples for hybridised, negative control (unhybridised) and blank were taken at time zero, cooled on ice for 1 min. and directly used for measurement of chemiluminescence using a dual injector Luminometer, by automatic injection of 200 µl of 0.4 M HNO_3, 0.1 % H_2O_2 then 200 µl of 1 M NaOH with a 1 second delay, followed by measurement of chemiluminescence for 5 seconds.

The reaction mixture tubes were removed at various time intervals (2-3 min) depending on the probe differential hydrolysis rate and assayed for chemiluminescence using the same the protocol as described above.

The data obtained were plotted as log of percent of remaining chemiluminescence versus time in minutes, where chemiluminescence at time zero was taken as 100 %. The values obtained from blank reaction mixture were subtracted to minimise signal to noise ratio for accurate measurement of hydrolysis time of the probes at the given pH. The hydrolysis rate of hybridised and unhybridised samples was calculated using standard regression analysis.

3.15 Optimisation of HPA assay

The HPA assay was optimised for efficient detection of target nucleic acid. The general HPA protocol described by Nelson *et al*., (1995) was used. Specific parameters such as volume of hybridisation buffer, time of hybridisation, pH of differential hydrolysis and time for differential hydrolysis were checked by changing a single parameter at a time, while remaining parameters were kept unchanged.

3.16 Optimised HPA protocol

Based on results obtained from preliminary experiments for optimisation of HPA the following protocol was used for carrying out further experiments, Several slightly modified protocols were also used as described in the specific sections.

3.16.1 Hybridisation

The hybridisation was carried out using 50 µl of succinate buffer 1 (0.1 M lithium succinate (pH 5.2), 2 mM EDTA, 2mM EGTA, 10 % lithium dodecyl sulphate (w/v)), 25 fmol of AE labelled DNA probe and 1 pmol of target nucleic acid were mixed in a 1.5 ml tube by vortexing. The negative control was set up in the same way without target molecules. The hybridisation was carried out by incubating the tube at 60 ºC for 45 min.

3.16.2 Differential hydrolysis

The differential hybridisation was carried out by adding 300 µl of 0.125 M sodium tetraborate (pH 8.0), 5 % Triton X-100. The tubes were incubated at 60 ºC for 7-10 min depending on differential hydrolysis kinetics of the particular probe. After incubation the tubes were placed on ice for 3 min.

3.16.3 Detection

The total volume from the differential hydrolysis step was taken into a polystyrene detection tube (Lumivials, Berthold) and chemiluminescence was measured using a dual injector Luminometer (Berthold model- LB-9706) by automatic injection of 200 µl of 0.4 M HNO_3, 0.1 % H_2O_2 then 200 µl of 1 M NaOH with a 1 second delay, followed by measurement of chemiluminescence for 5 seconds. The values were expressed in Relative Light Units (RLUs) (Nelson *et al*., 1995).

3.17 Additional HPA protocols

HPA assay involving detection of amplified DNA required an additional step of denaturation of double stranded DNA (Mullis and Faloona., 1987; Nelson and McDough., 1990, Nelson *et al*., 1995). For such assays 25 µl aliquot of amplified PCR product was transferred to a 1.5 ml tube and heated at 95 ºC for 10 min and cooled on ice for 3 min, 25 µl 2X Hybridisation buffer (0.2 M lithium succinate (pH 5.0), 4 mM EDTA, 4 mM EGTA, 0.8 M lithium chloride, 10 % lithium dodecyl sulphate (w/v)) was added to the tube. Hybridisation was carried out by incubating at 60 ºC for 60 min. The differential hydrolysis step was carried out by addition of 0.15 M sodium tetraborate, 5 % Triton-X 100 or 0.6 M boric acid 5 % Triton-X 100 (pH- 8.0), to the tube and incubating at 60 ºC for 7-8 min. Detection was carried out as described in section 3.16.3.

3.18 Slot blot analysis of RNA using DIG labelled probes

3.18.1 Slot blotting of RNA samples

Total RNA was serially diluted to the desired concentration using sterile deionised water and the samples were incubated at 72 ºC for 10 minutes to denature the RNA. The serial dilutions of total RNA were blotted on a nylon membrane (Hybond™), pre-wetted with 20X SSC using a **slot blot apparatus** (Schleicher & Schuell). The total cellular RNA was fixed on the nylon membrane using a UV crosslinker (UVC-508, Anachem, 1200,000 µJ of UV).

3.18.2 Prehybridisation and Hybridisation

Pre-hybridization was carried out for 30 minutes at 60 ºC with 10 ml of hybridization buffer (6 M Urea, 6X SSC, 1% (w/v) SDS and 100mM Tris-HCl, pH-7.0; Alzwiy and Morris, 2007). After pre-hybridization, 10 pmol/ml (final concentration) of DIG labelled probe was added and hybridization was carried out overnight at 60 ºC.

3.18.3 Stringency washing

After hybridization, the blot was washed in 2X SSC, 0.1 % SDS (low stringency) four times, 15 min each time, followed by washing with 0.2X SSC, 0.1 % SDS (high stringency) at 60 ºC two times for 10 minutes each time. After washing, the membrane was briefly immersed in DIG-1 buffer (0.1 M Tris-HCl, 1 M NaCl, 0.2 % Tween-80, pH- 8.5).

3.18.4 Blocking

The blot was placed in blocking solution (0.5 % blocking reagent Hammerstein casein, 0.1 M maleic acid, 350 mM NaOH, 1 M NaCl, 0.2 % Tween - 80, pH-8.0) and incubated at room temperature for 60 minutes. Anti-DIG conjugated to alkaline phosphatase (Roche) was diluted to 1:20000 in blocking buffer and incubated with the membrane for 30 minutes at room temperature. The membrane was washed with DIG-1 buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH- 8.5) buffer four times (10 minutes each time) and finally rinsed with DIG-4 buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH- 9.5) for 2-5 minutes.

3.18.5 Detection

Alkaline substrate solution (1 ml) (CDP Star, Sigma) was added to the membrane, placed between two sheets of transparent film and X ray film was used to detect the resulting chemiluminescence signal. Developer and fixer (Kodak) were used for X ray film development according to manufacturer's instructions in a dark room. The exposure time varied from 15 min to 2 hours depending on the intensity of signal.

3.19 Determination of specificity of AE probes

The specificity of AE probes was determined for closely related beer spoilage microorganisms as shown in Table 3.1. Hybridisation was carried out using 100 µl of succinate buffer 2 (0.1 M lithium succinate (pH 5.0), 1.5 mM EDTA, 1.5mM EGTA, 0.4 M lithium chloride, 5 % lithium dodecyl sulphate (w/v)), 0.1 pmol of AE labelled DNA probe and 1 pmol of target nucleic acid mixed in a 1.5 ml tube by vortexing. The negative control was set up in the same way without target RNA. Hybridisation was carried out by incubating the tube at 60 ºC for 45-60 min. Differential hydrolysis was carried out by adding 300 µl of 0.6 M boric acid (pH 8.0), 5 % Triton X-100. The tubes were incubated at 60 ºC for 7-10 mins depending on differential hydrolysis kinetics of the particular probe. After incubation the tubes were placed on ice for 3 min. The detection was carried out as described in section 3.16.3.

3.20 Determination of sensitivity of AE probes

AE labelled DNA probes developed for detection of *Pectinatus* and *Megasphaera* at genus and species level were use in the Hybridisation Protection Assay (HPA). Serially diluted bacterial RNA from target species was used for HPA assay using protocol

described in section 3.16. A negative control was maintained without target RNA and the blank reaction mixture contained only hybridisation buffer-2 without target nucleic acid and AE DNA probe.

3.21 Bacterial cell lysis for HPA assay

Three individual cell lysis procedures were used to obtain crude RNA extract from lysed bacterial cells and used directly for different HPA assays. A single bacterial colony was picked from a PYF agar plate and inoculated into 10 ml of PYF broth and incubated anaerobically for 12-18 hours. The concentration of viable bacterial cells (CFU/ml) was determined by plating 100 µl of serially diluted bacterial culture on a PYF agar plate and incubating anaerobically for 4-5 days at 30 ºC.

One ml aliquots of undiluted bacterial culture were centrifuged at 13000 rpm for 10 min and the pellet was frozen using liquid nitrogen and stored at -70 ºC till further use. Some aliquots of bacterial cultures were treated in the same way except an additional bacterial stabilisation procedure was followed as described in section 3.10.2.

3.21.1 Lytic enzyme solution

A pellet obtained from freezing 1 ml of bacterial culture was resuspended in sterile deionised water to obtain approximately 1x 10^8 cells/ml and vortexed vigorously to form a uniform suspension. The suspension was later fivefold serially diluted using sterile deionised water.

200 µL of cell lysis buffer consisting of TE buffer (30 mM Tris, 1 mM EDTA, pH 8.0), lysozyme (15 mg/ml) and 20 µL Proteinase K (Qiagen) was made and Ten µl of serially diluted sample was mixed with 90 µl above mentioned lytic enzyme buffer and incubated at 65 ºC for 10 min and cooled on ice for 3 min. Then 5-10 µl of lysed bacterial suspension prepared from known number of bacteria was used for HPA assay as crude RNA cell lysate using the HPA protocol as described in section 3.12

3.21.2 Detergent lysis protocol

Detergent based cell lysis buffer (Qiagen Puregene kit) and 1 % SDS were used for cell lysis. A pellet obtained from freezing 1 ml of bacterial culture was resuspended in sterile deionised water to obtain approximately 1x 10^8 cells/ml and vortexed vigorously to form a uniform suspension. The suspension was later fivefold serially diluted using sterile deionised water. 10 µl of serially diluted sample was mixed with 90 µl detergent based cell lysis buffer and incubated at 85 ºC for 10 min, cooled on ice for 3 min and

centrifuged briefly to precipitate cell debris. A 5-10 µl aliquot of supernatant was used as crude RNA for the HPA assay as described in section 3.16.

3.21.3 Heat lysis protocol

Frozen cell pellet or fresh bacterial pellet with was suspended in 0.1 % SDS to obtain 1 X 10^{8} cells per ml and fivefold serially diluted using sterile deionised water. A 50 µL aliquot of serially diluted cell suspension was heated at 95 ºC for 10 mins; snap cooled on ice for 3 mins and briefly centrifuged to precipitate cell debris. Lysed bacterial suspension (5-10 µL) of was used for HPA assay as crude RNA cell lysate using HPA protocol-1 as described in section 3.16.

3.22 Application HPA assay in brewing laboratory

Limit of detection of CFU using HPA assay was determined. The frozen pellet obtained (as described in section 3.21) with known CFU/ml was fivefold serially diluted and 10 µl aliquot of serially diluted sample was mixed with 90 µl detergent based cell lysis buffer and incubated at 85 ºC for 10 mins, cooled on ice for 3 mins and centrifuged briefly to precipitate cell debris. A 5-10 µl aliquot of supernatant was used as crude RNA for the HPA assay.

3.22.1 Determination of background noise from different beers

Various beer samples were obtained from local supermarkets and checked for background noise using the HPA protocol described in section 3.16.3. For each, 10 ml of beer sample was degassed by vigorous shaking in a conical flask for 15 mins and 5 µL of beer was added to 50 µL of hybridisation buffer-1 and the detection was carried out using HPA protocol as describe in section (3.16). The negative control was set up by using 5 µL of sterile deionised water.

3.22.2 Sensitivity of HPA assay in beer samples and beer enriched samples

Overnight grown bacterial cultures were fivefold serially diluted and bacterial colony forming units/ml were determined by plating 100 µl diluted suspension on PYF agar followed by incubation anaerobically at 30 ºC for 4-5 days. A 10 µl aliquot of serially diluted sample was mixed with 90 µl detergent based cell lysis buffer and incubated at 85 ºC for 10 min, cooled on ice for 3 min and centrifuged briefly to precipitate cell debris. A 5-10 µl aliquot of supernatant was used as crude RNA for the HPA assay.

100 ml samples of different beers (0% ABV, 2 % ABV, 4 % ABV and 6.6 % ABV) were spiked with a known number of bacterial cells (1-100 CFU/ml) and incubated at 30 ºC anaerobically. Similar experiments were set up to determine the effect of enrichment of beer with different media recommended for detection of *Pectinatus* and *Megasphaera*. MRS, NBB and SMMP medium were used for enrichment of beer. MRS (de Man *et al.,* 1960) and NBB (Kindraka, 1987) media were mixed with different beers (0% ABV, 2 % ABV, 4 % ABV and 6.6 % ABV) in 1:1 ratio (v/v) while SMMP medium (Lee, 1994) was used as recommend by (Dull *et al.,* 1998) (15 % medium component: 85 % beer). Final volumes of 100 ml were used in duplicate. A negative control was maintained for each combination without inoculation. One ml was removed from the samples at intervals of 24 hours and the sample was centrifuged at 13000 rpm for 3 minutes to collect the cells. The cell pellet was washed with sterile deionised water and suspended in 10µL deionised water and 90 µL cell lysis buffer (Qiagen) and vortexed briefly to dissolve the cell pellet. The sample was incubated at 85 ºC for 10 mins, cooled on ice for 3 mins and centrifuged briefly to precipitate cell debris. A 5-10 µl aliquot of supernatant was used as crude RNA for the HPA assay as described in section 3.16.

3.23 Analysis of real brewery samples

The HPA assay developed was applied for detection of putative isolates of *Pectinatus* and *Megasphaera* from brewery environments and the same samples were analysed using SMMP medium (Lee, 1994; Dull *et al.,* 1998), multiplex PCR method (Asano *et al*., 2008, Suzuki *et al*, 2008), RNA slot blot analysis and partial 16S rRNA gene sequencing for comprehensive comparison of detection methods used.

3.24 Statistical Analysis

The DH kinetics was carried out using standard regression analysis as stated previously by Nelson *et al*., (1995). The one way ANOVA ($\alpha = 0.05$) followed by Dunett's test (using Sigma Plot version 11.0) carried out to compare effect of different cell lysis protocols on the HPA assay. The HPA assay data are represented as mean RLUS or Mean S/N ratio of five replicates, unless otherwise stated.

CHAPTER: 4 RESULTS

PART-I

Chapter: 4 RESULTS

Results Part I Occurrence of *Pectinatus* and *Megasphaera* in UK breweries

4.1 Optimization of multiplex PCR methodology

Multiplex PCR reactions were set up in four reaction formats for each of the three *Pectinatus* species (*Pectinatus* multiplex), three *Megasphaera* species (*Megasphaera* multiplex), six main beer spoilage *Lactobacillus* species (*Lactobacillus* multiplex) and three *Pediococcus* species as previously described by Asano *et al.* (2008) and Ijima *et al.*(2008). All primers were based on rRNA gene sequences and in some species the internal transcribed spacer (ITS) region. List of primers used for the present study are shown in Tables 3.2 and 3.3. Optimisation of the multiplex PCR method was carried out according to a stepwise protocol described by Henegariu *et al.,* (1997). PCR reactions were optimized and successfully used for further detection of real brewery samples. The original multiplex protocols (Asano *et al.,* 2008; Ijima *et al.,* 2008) comprised of 30 cycles of denaturation, annealing and extension (15, 15 and 30 seconds respectively) which was modified to 30 cycles of 30 sec, 30 sec and 1 min respectively for all four multiplex PCR methods. For all four PCR regimes, equimolar concentration of primers (100 pmol/µl) was used.

It was verified that *Pectinatus, Megasphaera, Lactobacillus* and *Pediococcus* species were detected with high specificity and selectivity. No internal positive control was established but external positive and negative controls were carried out for each batch of PCR using DNA extracted from *P. cerevisiiphilus, M. cerevisiae* and *L. brevis* for *Pectinatus*, *Megasphaera* and *Lactobacillu*s multiplex PCR.

The multiplex PCR was checked for any inhibition by commercial lager which was used for enrichment of swab samples and no specific protocol was followed to remove PCR inhibitory substances from the samples except that the cell pellet was repeatedly washed with deionised water. However the DNA isolation procedure includes ethanol precipitation of DNA which is effective for removal of inhibitory substances in the beer. PCR inhibitory substances are however a real concern when crude cell lysis suspension is used directly for PCR reactions.

4.2 Specificity of Multiplex PCR

The specificity of all PCR regimes was checked using 1 µg (500-700 ng/µl) of DNA isolated from target and closely related organism as shown in Table 3.1. For each reaction an equimolar concentration of primers (100 pmol) was used.

All of the four multiplex PCR primers (Table 3.3 and 3.4) showed high specificity towards the target bacterial species and none of the PCR runs showed amplification of non-specific bands. The ability of all the four multiplex PCR formats to amplify target DNA, in mixed format was also evaluated, and the results are illustrated in the Figure 4.1. The figure shows successful amplification of DNA from target organisms in individually and mixed formats.. The specificity for the primers used in *Pectinatus* and *Megasphaera* multiplex were checked at different primer annealing temperatures (54, 55, 56 º C), and the result showed no loss in the specificity of the species specific primers (data not shown)

4.3 Sensitivity of multiplex PCR

Sensitivity of the *Pectinatus* multiplex PCR was found to be within the range of 10-100 fg of target DNA. For *Pectinatus* PCR, *P. cerevisiiphilus* and *P. frisingensis* showed successful amplification at 10 fg of target DNA whereas sensitivity of *P. haikarae* was limited to ≥ 100 fg. *Megasphaera* multiplex was found to be sensitive enough to detect ≤ 100 fg of target DNA; however for *M. cerevisiae* 10 fg of target DNA could be successfully amplified, whereas the sensitivity to amplify target genomic DNA of *M. paucivorans* was limited to ≤ 100 fg.

For *Lactobacillus* multiplex, the primers for *L. brevis* and *L. lindneri* showed different sensitivity by detecting 10 fg and 100 fg of target DNA respectively. Sensitivity of PCR for *L. casei*, *L. paracollinoides* and *L. plantarum* was not determined. In implementation of previously described protocols for beer spoilage cocci, *Pediococcus* and *Megasphaera* species (Ijima *et al*., 2008) showed reduction in sensitivity of *Ped. damnosus* to > 100 fg, hence for the present study *Pediococcus* multiplex was independently implemented, the details of primers used are given in Table 3.3.

For the developed *Pediococcus* multiplex, *Ped. damnosus* was detected at a target DNA concentration of ≤ 100 fg. For all the PCR reactions, positive results were concluded based on visibility of an appropriate sized amplified band on agarose gel. For simplex PCR, a sensitivity of 10 fg has been reported previously (Juvonen, 2009). The PCR methods used in this study showed sensitivity to detect target DNA concentration of ≤ 100 fg. The sensitivity of all four PCR formats using serially diluted DNA from target organisms is shown in the Figure 4.2.

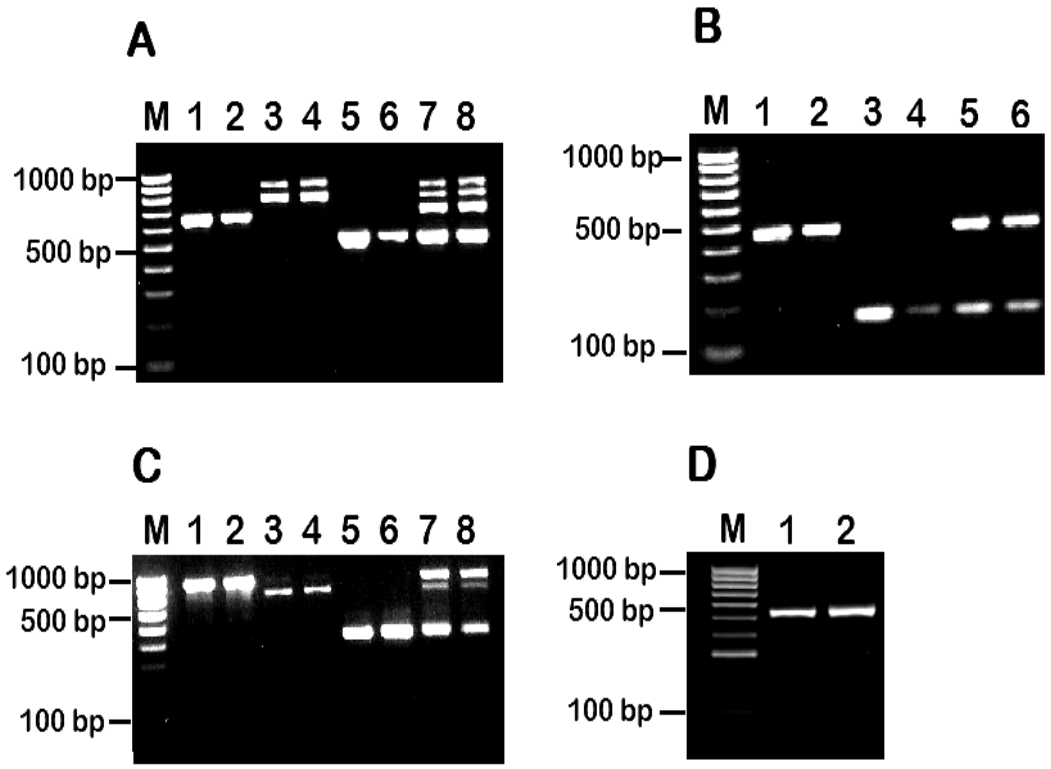


Figure 4.1 Specificity of PCR multiplex primers. **A:** *Pectinatus* multiplex PCR was examined for specificity using *P. cerevisiiphilus* (1, 2), *P. frisingensis* (3, 4), *P. haikarae* (5, 6) and all three *Pectinatus* species (7, 8). **B:** *Megasphaera* multiplex PCR was carried out using different combinations of target bacterial species *M. cerevisiae* (1, 2) *M. paucivorans* (3, 4), *M. cerevisiae and M. paucivorans* (5, 6). **C**: *Lactobacillus* multiplex was partially checked for specificity using *L. brevis* (1, 2), *L. lindneri* (3, 4*), L. plantarum* (5, 6) and (7,8) *L. brevis, L, lindneri* and *L. plantarum*; **D** *Pediococcus* multiplex PCR, amplified fragments for, 1- *Ped. damnosus* (1) and 2- *Ped. inopinatus* (2). M represents 100 bp DNA ladder (Hyper ladder IV, Bioline).

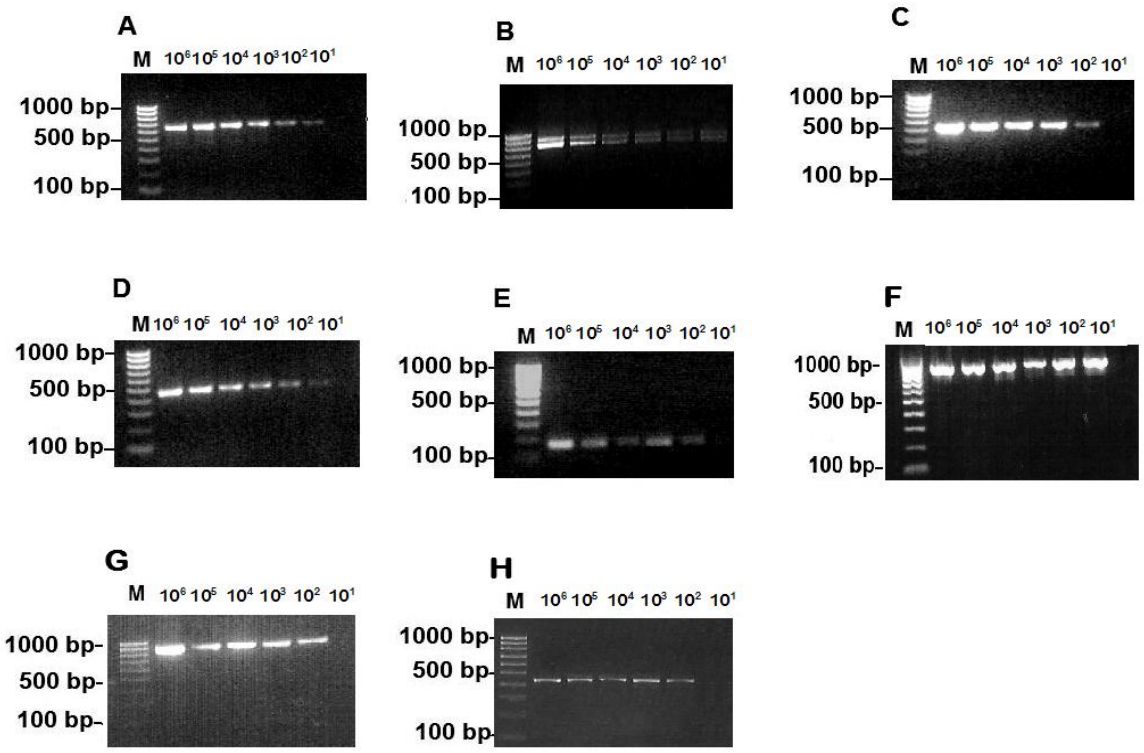


Figure 4.2 Sensitivity of *Pectinatus*, *Megasphaera*, *Lactobacillus* and *Pediococcus* multiplex PCR. **A**, **B** and **C** show PCR amplified fragments for 10 fold serial dilution of DNA template isolated from *P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae*. **D** and **E** show multiplex PCR fragments amplified using serially diluted DNA isolated from *M. cerevisiae* and *M paucivorans* respectively; **F**, **G** and **H** show amplification results for *L. brevis*, *L. lindneri* and *Ped. damnosus* respectively. In every figure 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 represent dilution of bacterial DNA (fg). M represents 100 bp DNA ladder (Hyper ladder IV, Bioline).

4.4 Multiplex PCR results

Collection and enrichment of samples were important tasks during the study. All the samples were treated on site soon after collection and anaerobic conditions were maintained during transportation of samples to the laboratory using an anaerogen kit (Merck) and anaerobic jar. PCR is a highly sensitive method for detection of even low levels of contaminants in samples but for detection of highly anaerobic bacteria, enrichment of samples was carried out for 14 days. For strict anaerobes culture enrichment is needed to achieve detectable numbers of cells in samples. In addition sometimes the high volume of sample is more important than incubation time to achieve detectable growth of target microorganisms (Juvonen *et al.*, 2008) and the volume of samples was 250 ml to overcome this limitation of enrichment method.

During the investigation of anaerobic beer spoilage bacteria in 10 major UK breweries, 117 samples were analysed. Of these 117 samples, two samples were positive for *P. cerevisiiphilus*; four samples were positive for the presence of *P. frisingensis*, two samples showed the presence of *M. cerevisiae* and one sample was found positive for the presence of *M. sueciensis* and *M. paucivorans* (detected by same pair of primers). PCR positive samples for *Pectinatus*, *Megasphaera*, *Lactobacillus* and *Pediococcus* (multiplexes) are shown Table 4.1. *L. brevis* and *L. lindneri* were found to be the most frequently occurring *Lactobacillus* species with 16 and 13 positive samples respectively, while *L. casei*, *L. plantarum* and *L. corynformis* were found in 3, 1 and 1 samples respectively. Ten actual beer samples were positive for the presence of *Lactobacillus* species mainly from conditioning areas and filtration units. *Pediococcus* multiplex resulted in 24 positive samples for *Ped. damnosus/ Ped inopinatus*. Out of 24 samples positive for *Ped. damnosus/ Ped. inopinatus*, three samples were found from direct beer samples and the remaining samples were found positive for indirect samples. Additionally two yeast samples were received from brewery 3; both the samples were found to be positive for *Ped. damnosus* and one sample was positive for the presence of *L. paracollinoides*. The identity of these samples was later confirmed using partial 16S ribosomal gene sequencing.

Samples from star wheels of bottling lines from breweries 1 and 3 were positive for the presence of *P. cerevisiiphilus*, while for *P. frisingensis*, two conveyor belt sterile swab samples from brewery 2 and two samples both from CO_2 collecting bubble pods of fermentors from brewery 5 were positive. All six positive samples for *Pectinatus* were from indirect sampling points and none of the isolates from direct beer samples were found to be positive. It was interesting to find that the samples from star wheels and conveyor belts samples which are highly aerobic in nature showed the presence of strictly anaerobic beer spoilage bacteria. Positive samples for *Pectinatus* and *Megasphaera* multiplex are illustrated in Figure 4.3

The liquid rinse samples from the CO_2 bubble pods were also interesting as the presence of *Pectinatus* species from the fermentation area is considered to be rare but the isolation of anaerobic beer spoilage bacteria from the CO_2 recovery systems has been frequently reported from breweries in UK (Binns, P., personal communication). Each sample from breweries 2 and 3 was positive for presence of *Megasphaera* species and one sample from brewery 2 taken from conveyor belt swab of the canning lines was positive for the presence of *M. paucivorans* and *M. sueciensis*.

None of the samples from other breweries 4, 6, 7, 8 9 and 10 showed the presence of *Pectinatus* or *Megasphaera* according to PCR analysis. On the other hand *Lactobacillus* and *Pediococcus* species were found to be distributed among samples from all breweries.

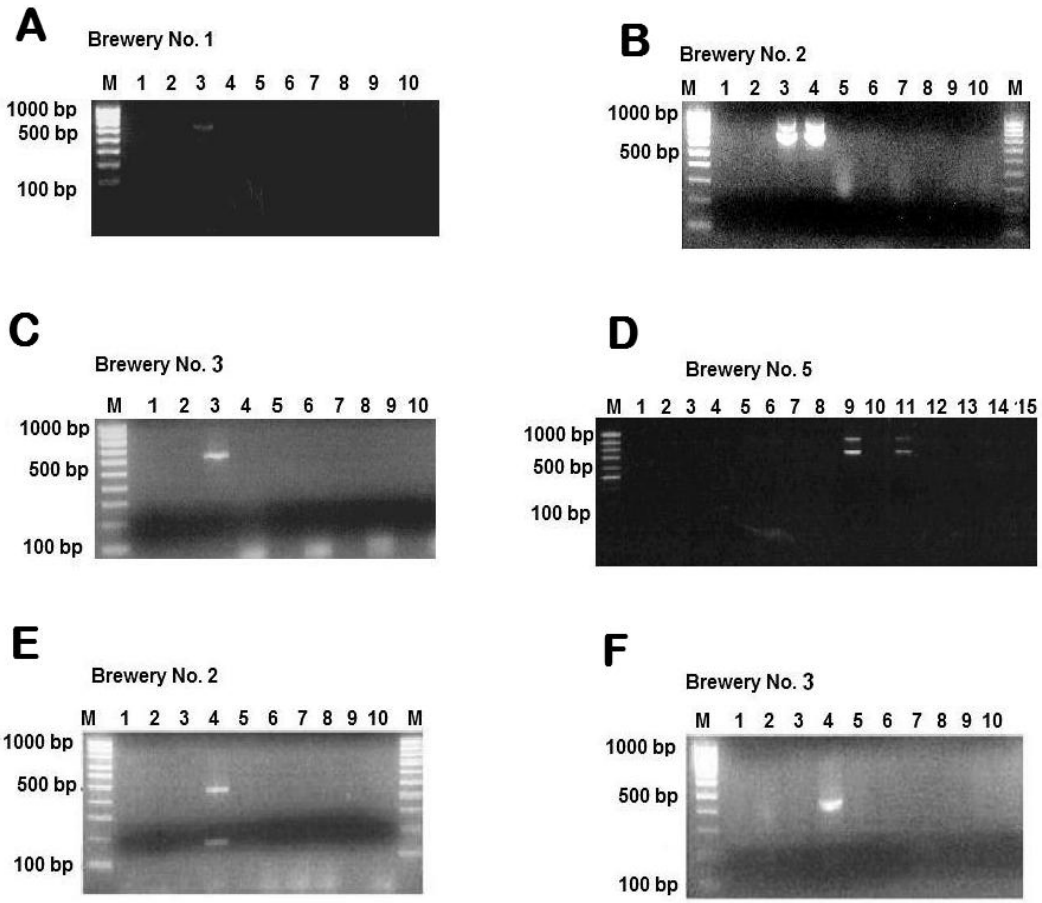


Figure 4.3 *Pectinatus* and *Megasphaera* multiplex PCR analysis of samples from breweries showing positive results. **A**, **B, C and D** represent *Pectinatus* multiplex results for brewery 1, 2, 3 and 5 respectively. Sample 3 from breweries 1 and 3 were positive for the presence of *P. cerevisiiphilus*. Samples 3 and 4 from brewery 2 and samples 9 and 11 from Brewery 5 were positive for the presence of *P. frisingensis*. **E and F** represent *Megasphaera* multiplex PCR results for brewery 2 and 3 respectively. Sample 4 from brewery 2 was positive for presence of *M. cerevisiae* and *M. paucivorans*/ *M. sueciensis.* Sample 4 from brewery 3 was positive for presence of *M. cerevisiae*. **M** represents 100 bp DNA ladder (Hyper ladder-IV, Bioline).

Table 4.1 Multiplex PCR results for the sampled breweries

	Brewery No.	1	2	3	4	5	6	7	8	9	10
	Total no. of samples	10	10	10	10	15	15	7	10	10	20
No. of positive samples through multiplex PCR	*P. cerevisiiphilus*	1[a]		1[a]							
	P. frisingensis		2[a]			2[a]					
	P. haikarae										
	M. cerevisiae		1[a]	1[a]							
	M. paucivorans & *M. sueciensis*		1[a]								
	L. brevis	5[ab]	3[ab]	2[a]	1[a]	2[a]	2[a]			1[b]	
	L. lindneri			2	3[a]			1[b]	1[b]	4[ab]	2[a]
	L. casei									1[b]	2[a]
	L. corynformis								1[b]		
	L. plantarum							1[b]			
	L. paracollinoides										
	Ped. damnosus	3[a]	2[a]	3[a]	4[a]	4[ab]	1[a]		1[b]	1[b]	5[a]
	Ped. inopinatus										
	Ped. claussenii										

a- Sample collected from indirect sampling points- swabs and rinse samples from vessels and packaging equipment.

b- Samples collected from direct beer samples- beer sample/ fermenting wort and yeast slurry.

Survival of strictly anaerobic bacteria in an aerobic environment can possibly be due to biofilm formation (Back, 1994; Suzuki 2011). Instruments used in the filling process are prone to formation of biofilms which are a niche for various beer spoiling microorganisms. The slime produced by these biofilms can protect microbes from routine cleaning procedures. Yeast and *Lactobacillus* species can dwell in these slimes, while lactic acid produced by *Lactobacillus* species can be metabolized to propionic acid by anaerobic bacteria such as *Pectinatus* species, which can cause undesirable changes to final products (Tholozan *et al.*, 1997). Detection of low levels of *Pectinatus* from biofilms on a conveyor belt in a beer bottling line based on fatty acid profiles has been previously reported (Timke *et al.*, 2005) . The presence of *Pectinatus* and *Megasphaera* species from fermentation areas and bottling lines of four major breweries in the UK (breweries 1, 2, 4 and 5) shows that *Pectinatus* and *Megasphaera* species are natural inhabitants of the breweries in the UK and not frequent invaders.

Table 4.2 Summary of hygiene monitoring, inspection and microbial methods utilized in the breweries

Brewery No.	Capacity (Hl)	Hygiene certification	Packaging facilities	Microbial detection methods/ anaerobic media used	Hygiene Inspection	CIP formulation used for packing Lines
1	190,0000	No data	bottling, canning kegging	Plate count method Raka Ray	No data	Automatic caustic CIP (1-2 %) twice weekly
2	400,0000	ISO 9001	canning kegging casking	Plate count method Raka Ray	ATP*	Automatic caustic CIP(1-2%) twice weekly
3	900,0000	ISO 9001	bottling canning kegging	Plate count method Raka Ray	ATP*	Automatic caustic CIP(1-2%) twice weekly
4	400,0000	ISO 9001	bottling kegging casking	Plate count method Raka Ray	No data	Automatic caustic CIP(1-2%) twice weekly
5	400,0000	ISO 9001	bottling canning kegging	Plate count method Raka Ray	ATP*	Automatic caustic CIP(1-2%) + combination of per acetic acid (PAA) and Chlorine(Cl_2) , Twice weekly
6	380,0000	BRC HACCP	bottling canning kegging	Plate count method Raka Ray NBBC broth	ATP*	Automatic acid CIP commercial formulation (Johnson Diversey), chemicals, UK

7	190,0000	ISO-9001 BRC	kegging	Plate count method Raka Ray	ATP*	Automatic caustic CIP (1-2 %) after every use
8	110,0000	ISO-14000 BRC	kegging	Plate count method Raka Ray	ATP*	Automatic caustic CIP twice weekly
9	110,0000	ISO-9001 ISO-22000 ISO-14000	kegging	Plate count method Raka Ray	ATP*	Automatic caustic CIP twice weekly
10	No data	BRC	bottling canning kegging	Plate count method Raka Ray NBBC broth	ATP*	Automatic caustic CIP every 48 hours / acid CIP occasionally

BRC- British Retail Consortium, HACCP- The Hazard Analysis and Critical Control Point certification, ISO- International Organization for Standardization

*- ATP bioluminescence based method using hand held and portable luminometer.

4.5 Hygiene monitoring and microbial methods used in the breweries

A summary of hygiene monitoring and microbial methods adopted in the breweries sampled at the time of this study is given in Table 4.2. In all 10 breweries, conventional microbiological practices are adopted for detection of beer spoilage contaminants based on plate count methods. For the detection of beer spoilage anaerobes, Raka Ray medium has been recommended by European Brewing Convention (EBC) (Sakamoto *et al.,* 2003) and this medium supplemented with cyclohexamide and 2-phenyl ethanol is utilized in all of the 10 breweries. In addition, two breweries utilise NBBC broth for detection of anaerobes. None of the breweries use SMMP medium (Selective Medium for detection of *Megasphaera* and *Pectinatus*) (Lee, 1994) for detection of *Pectinatus* and *Megasphaera* in brewery samples. Raka Ray medium has the limitation of detecting only facultatively anaerobic bacteria belonging to *Lactobacillus* species and recovery rate on this medium is not good (Quain, D., personal communication) hence it can be confirmed that except for NBBC, no effective medium is utilized to specifically detect *Pectinatus* and *Megasphaera* in the UK breweries.

The identification of brewery contaminants is mainly based on microscopic analysis. Thus it can be concluded that microbial spoilage due to anaerobic bacteria cannot be specified by the conventional methods used in these breweries unless NBBC is used. Raka Ray medium is also not sufficient to detect hard to grow lactic acid bacteria such as *L. lindneri*, *L. paracollinoides* and *Ped. damnosus* (Suzuki *et al*., 2008). Cleaning and hygiene validation of fermentation tanks, beer storage tanks and packaging lines is carried out by using an ATP bioluminescence method in 8 out of the 10 breweries.

At present automated CIP (Cleaning in Place) with varying concentration of sodium hydroxide (NaOH; 1-2 %), cold and hot CIP, once or twice a week is utilised in most of the breweries (Table 4.2). In general, filling equipments are cleaned using automated caustic CIP and foam cleaning after every use. Brewery 6 utilizes an acid based commercial formulation (Johnson Diversey Chemicals, UK) and this brewery showed comparably better hygienic conditions in brewery equipment and canning lines with none of the samples being positive for *Pectinatus* and *Megasphaera* and only two samples positive for *Lactobacillus* and one sample from an indirect sampling point positive for *Ped. damnosus/ Ped. inopinatus.* Brewery 5 utilizes disinfectants such as PAA (per-acetic acid) and Cl_2 (chlorine) in addition to caustic CIP for cleaning of bottling and canning lines respectively.

CHAPTER: 3 RESULTS

PART- II

Results Part II Characterisation of putative isolates of Pectinatus and *Megasphaera*

4.6 Isolation of *Pectinatus* and *Megasphaera* from brewery samples

Putative isolates of *Pectinatus* and *Megasphaera* were isolated as described in section 2.6. Both the putative *P. cerevisiiphilus* isolates from the respective samples from brewery 1 and 3 were successfully isolated in pure culture. Out of four *P. frisingensis* positive samples only three isolates were successfully retrieved in pure form. Sample 4 from brewery 3 was positive (using multiplex PCR) for *M. cerevisiae* and (*M. paucivorans/ M. sueceinsis*), but only *M. cerevisiae* isolates were successfully retrieved while *M. paucivorans/ M. sueceinsis* failed to culture. From brewery 2 a putative isolate of *M. cerevisiae* was also successfully cultured. In addition one putative *M. cerevisiae* isolate was obtained from brewery 5, isolated from a rinse sample collected from a CO_2 recovery system. Over-all two putative isolates of *P. cerevisiiphilus*, three isolates of *P. frisingensis* and three isolates of *M. cerevisiae* were studied. The details of the isolates are shown in Table 4.3

Table 4.3 Details of bacterial isolates

Putative isolates	Brewery No.	Generic identity
P. cerevisiiphilus	1	ICBD strain PC-1
P. cerevisiiphilus	3	ICBD strain PC-2
P. frisingensis	3	ICBD strain PF-1
P. frisingensis	5	ICBD strain PF-2
P. frisingensis	5	ICBD strain PF-3
M. cerevisiae	2	ICBD strain MC-1
M. cerevisiae	3	ICBD strain MC-2
M. cerevisiae	5	ICBD strain MC-3[1]

1- additional isolate of putative *M. cerevisiae* obtained from brewery 5

4.7 General physiological characterisation

The general characteristics of putative isolates of *Pectinatus* and *Megasphaera* are illustrated in Table 4.4. All the isolates of *Pectinatus* were found to be Gram negative, oxidase negative and catalase negative. Growth of the isolates ranged within the pH 4-8, with exception of ICBD strain PF-1 and PF-3, which were able to grow at pH 8.5. Putative *P. cerevisiiphilus* strains (ICBD strains PC-1 and PC-2) were able to grow at a temperature of 45 ºC, whereas remaining isolates of *Pectinatus* and *Megasphaera* were able to grow within the temperature range 10-37 ºC.

The ICBD strains PC-1 and PC-2 showed weak acid production during glycerol and mannitol utlisation, whereas the culture collection strain *P. cerevisiiphilus* (DSM 20467) was negative for acid production for glycerol utilisation. None of the *Pectinatus* isolates were able to utilise D-lactose or sucrose for acid production. The detailed sugar utilisation profiles of isolates of *Pectinatus* are shown in Appendix II. No major difference was found in sugar utilisation profiles of *Pectinatus* isolates and the corresponding culture collection strains. The sugar utilisation profiles for putative isolates of *Megasphaera* were not determined.

4.8 Morphological characterisation

All the isolates showed moderate growth on PYF, MRS and SMMP agar. The colonies of putative *P. cerevisiiphilus* isolates (PC-1 and PC-2) on PYF and MRS media appeared roughly circular, shiny, concave, creamy yellow, with entire margin while on SMMP agar colonies appeared relatively smaller, irregular and violet-blue in colour due to accumulation of crystal violet stain around the colonies. Similarly putative isolates of *P. frisingensis* (ICBD strain PF-1, PF-2 and PF-3), when grown on PYF medium appeared relatively large, round and creamy with shiny textures but on SMMP agar these isolates appeared moderate, concave to pyramidal with undulate margin. Putative strains of *M. cerevisiae* (ICBD strains MC-1, MC-2 and MC-3) on PYF and MRS appeared small, round, glossy and mucoid with entire margin. The growth on SMMP medium showed change in colour of medium from violet-blue to yellow within 7 days of inoculation (Figure 4.4). Change in colour of the SMMP agar has been previously documented for brewery isolates of *M. cerevisiae* (Dull *et al.*, 1998).

Table 4.4 General physiological characteristics of 8 putative strains of *Pectinatus* and *Megasphaera* isolates

Characteristics	**Bacterial strains and isolates**													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Gram staining	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mobility	+	+	+	-	-	-	+	+	+	+	+	-	-	-
Catalase test	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temp. range (°C)	10- 45	10- 37	15- 30	10- 37	10- 30	10- 30	10- 45	10- 45	10- 37	10- 37	10- 37	10- 37	10- 37	10- 37
pH range	4-8	3.5-8	4-8	4-8	4-8	4-8	4-8	4-8	4-8.5	4-8	4-8.5	4-8	4-8	4-8
Acid production														
glycerol	w	-	-	ND	ND	ND	+	w	+	+	w	ND	ND	ND
Esculin	+	+	-	ND	ND	ND	+	+	+	w	w	ND	ND	ND
D- lactose	-	-	+	ND	ND	ND	-	-	-	-	-	ND	ND	ND
D-xylose	+	-	+	ND	ND	ND	+	+	-	-	-	ND	ND	ND
Mannose	+	+	+	ND	ND	ND	+	+	+	+	+	ND	ND	ND
Mannitol	-	+	+	ND	ND	ND	w	w	+	+	+	ND	ND	ND
Sucrose	-	-	-	ND	ND	ND	-	-	-	-	-	ND	ND	ND

1- *P. cerevisiiphilus* (DSM-20467); 2- *P. frisingensis* (DSM-6306; 3- *P. haikarae* (DSM 16980); 4- *M. cerevisiae* (DSM 20461); 5- *M. sueceinsis* (DSM-17042); 6- *M. paucivorans* (DSM 16981); 7- *P. cerevisiiphilus* (ICBD strain- PC -1), 8- *P. cerevisiiphilus* (ICBD strain- PC -2; 9- *P. frisingensis* (ICBD strain- PF-1; 10- *P. frisingensis* (ICBD strain- PF-2); 11- *P. frisingensis* (ICBD strain- PF-2); 12- *M. cerevisiae* (ICBD strain- MC-1); 13- *M. cerevisiae* (ICBD strain- MC-2; 14- *M. cerevisiae* (ICBD strain- MC-3)

(-) negative result, (+) positive results, w – weakly positive, ND- not determined.

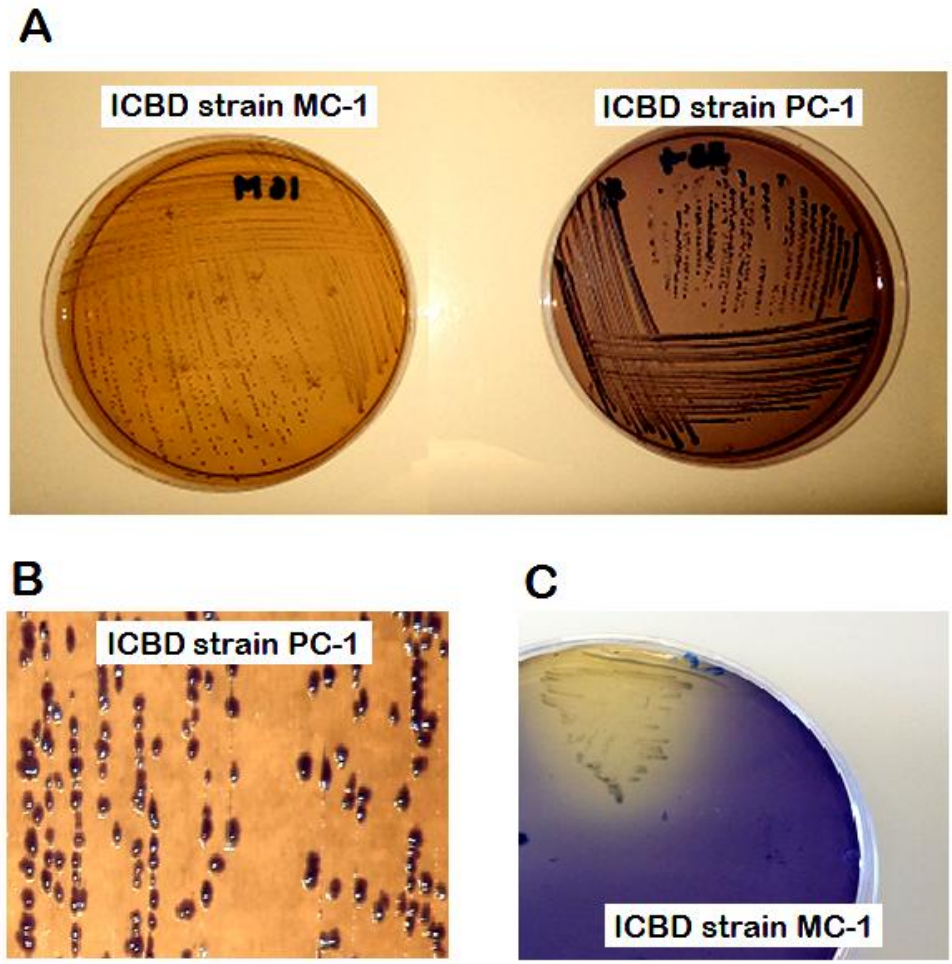


Figure: 4.4 Characteristics of brewery isolates **A:** Comparison of growth of the putative *Pectinatus* and *Megasphaera* isolates on SMMP agar when incubated under anaerobic conditions at 30 ºC for 7 days. The change in colour from violet to yellow of medium can be clearly seen for putative *M. cerevisiae* isolate (ICBD strain MC-1) but the growth of putative *P. cerevisiiphilus* strain (ICBD strainPC-1) has minimal effect on colour of the agar. **B:** Putative *P. cerevisiiphilus* isolate (ICBD strain PC-1) colonies on SMMP agar; the colonies appear violet-blue in colour due to accumulation of crystal violet. **C:** Putative *M. cerevisiae* isolate (ICBD strain MC-1) on SMMP agar, the change in colour from violet to yellow of medium can be clearly seen in putative *M. cerevisiae* isolate.

4.9 Scanning electron microscopy (SEM)

SEM images were obtained for the ICBD strains PC-1, PC-2, PF-1, PF-2, MC-1 and MC-3. The beer spoilage *Pectinatus* species were Gram negative, non spore forming and straight to curved rods. The most unique feature of beer spoilage *Pectinatus* species is a comb like arrangements of flagella on one side of the cell (Lee *et al*., 1978; Juvonen, 2009). The ICBD strain PF-1 as shown in Figure 4.5, shows presence of flagella, whereas in SEM images of ICBD strain PC-1 the arrangements of flagella were not visible in the SEM micrographs. All three beer spoilage *Megasphaera* species were cocci, non-spore forming and non motile, mainly appearing in pairs and rarely in short chains. The arrangement of cells in pairs (diplococci) can be clearly seen, for ICBD strains MC-1and MC-3

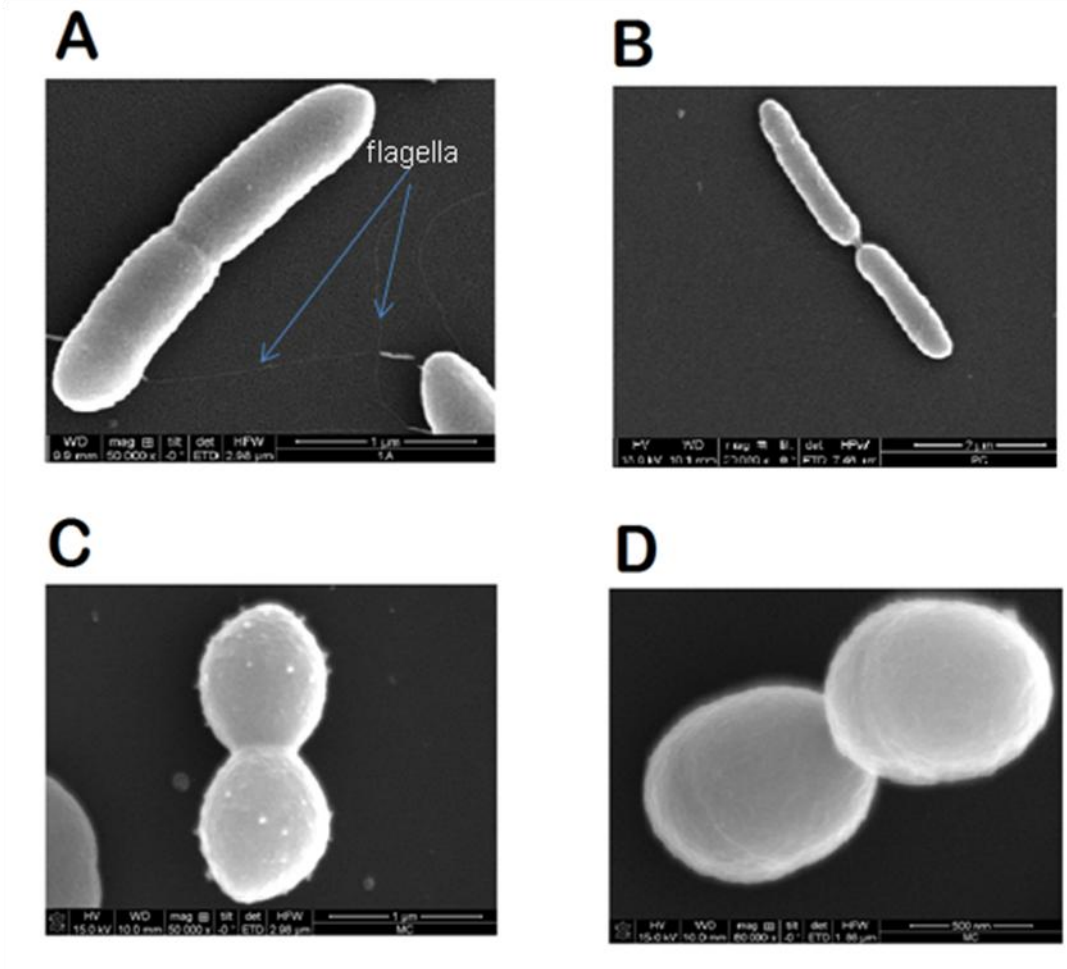


Figure: 4.5 SEM images of putative isolates of *Pectinatus* and *Megasphaera*. **A** and **B** represent SEM images of putative *Pectinatus* isolates (ICBD strain PF-1 and ICBD strain PC-1) respectively. **C** and **D** represent SEM images of putative *Megasphaera cerevisiae* isolates (ICBD strains MC-1 and MC-3 respectively).

4.10 Antibiotic susceptibility

All the putative isolates of *Pectinatus* and *Megasphaera* were checked for antibiotic susceptibility using MAST ID MID- 8 rings (MAST Diagnostic, UK). The antibiotic susceptibility of culture collection strains of *Pectinatus* and *Megasphaera* used in this study were also examined. All the culture collection strains and the putative isolates of *Pectinatus* and *Megasphaera* were clearly resistant to Penicillin G (2 units); similar results have been previously documented for culture collection strains (Zhang *et al*., 2012). All the putatative *Pectinatus* and *Megasphaera* strains were found to be susceptible to kanamycin (1000 µg) and colistin sulphate (10 µg), similar to results that have been obtained previously for beer spoilage culture collection strains of *Pectinatus* and *Megasphaera* (Juvonen and Suihko, 2006; Zhang *et al*., 2012). It was interesting to note that all the putative isolates of *Pectinatus* and *Megasphaera* along with culture collection strains used in the study showed strong susceptibility to erythromycin (60 µg) and weak susceptibility to rifampicin (15 µg). Except for *P. cerevisiiphilus* (DSM 20467) and putative *P. cerevisiiphilus* isolates (ICBD strain PC-1 and PC-2) all the strains were resistant to vancomycin (5 µg). The summary of antibiotic susceptibility is shown in Table 4.5. A comparison between M. cerevisiae (DSM 20461) and ICBD strain MC-1 is shown in Figure 4.6.

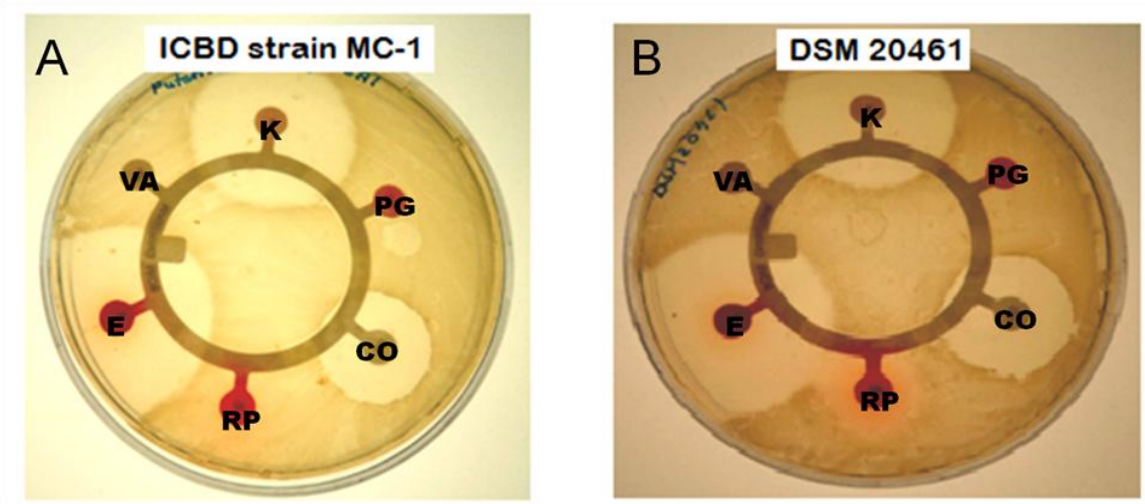


Figure: 4.6 A comparison of antibiotic susceptibility profile of putative *M. cerevisiae* isolate (ICBD strain MC-1) and *M. cerevisiae* culture collection strain (DSM 20461). Both the strains are clearly resistant to Penicillin G (2 units) and vancomycin (5 µg), while similar susceptibility profile between the strains can be seen for other antibiotics examined. K= kanamycin, PG= Penicillin G, Co= colistin sulphate, RP= rifampicin, E= erythromycin and VA= vancomycin in both the figures.

Table 4.5 Results for antibiotic susceptibility test using MAST ID MID-8 ring

S.N	Antibiotic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Erythromycin (60 µg)	++	++	+++	+++	+++	+++	++	++	+++	+++	++	++	+	++
2	Rifampicin (15 µg)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Colistine sulphate (10 µg)	++	+	+++	++	+	++	++	++	+++	+++	++	++	++	++
4	Penicillin G (2 units)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Kanamycin (1000 µg)	+++	+	+++	+++	+++	+++	+	++	+++	+++	+++	++	++	++
6	Vancomycin (5 µg)	+	-	-	-	-	-	+	+	-	-	-	-	-	-

+ Susceptible, ++ moderate susceptibility, +++ high susceptibility, - Resistant

1- *P. cerevisiiphilus* (DSM-20467); 2- *P. frisingensis* (DSM-6306); 3- *P. haikarae* (DSM 16980); 4- *M. cerevisiae* (DSM 20461); 5- *M. sueceinsis* (DSM- 17042); 6- *M. paucivorans* (DSM 16981); 7- *P. cerevisiiphilus* (ICBD strain- PC -1), 8- *P. cerevisiiphilus* (ICBD strain- PC -2; 9- *P. frisingensis* (ICBD strain- PF-1; 10- *P. frisingensis* (ICBD strain- PF-2); 11- *P. frisingensis* (ICBD strain- PF-3); 12- *M. cerevisiae* (ICBD strain- MC-1); 13- *M. cerevisiae* (ICBD strain- MC-2; 14- *M. cerevisiae* (ICBD strain- MC-3)

From the results obtained in this study, this suggests that the combination of antibiotic susceptibility using the disc diffusion method with other conventional methods can be effectively used to identify putative strains of *Pectinatus* at species level. Based on susceptibility to vancomycin (5 µg), *P. cerevisiiphilus* which is susceptible to vancomycin can easily distinguished from vancomycin resistant *P. frisingensis* and *P. haikarae*. Further these two species can be distinguished based on the catalase test as *P. haikarae* is positive for the catalase test whereas *P. frisingensis* and *P. cerevisiiphilus* are catalase negative.

P. cerevisiiphilus (DSM 20467) and putative isolates were found to be susceptible to vancomycin. Susceptibility of *P. frisingensis* to nisin has also been documented; vancomycin and nisin are molecules are relatively large in size (Suzuki, 2011) and normally unable to penetrate through outer membrane of Gram negative bacteria (Chihib *et al*., 1999). In contrast hop compounds resistance of *Pectinatus* and *Megasphaera* has been supposed to be due to thick outer membrane and multiple efflux system (Haikara and Helender, 2004). The outer membrane of these microorganisms may not the possible reason for hop resistance or it may be suggested that *Pectinatus* species posses complex resistance system for hop bitterness compounds along with effective efflux system (Suzuki, 2011)

4.11 Determination of beer spoilage ability

A 500 µL aliquot of overnight grown bacterial culture was added to 100 ml degassed beer and incubated anaerobically at ºC for 14 days. The bacterial growth was monitored using a spectrophotometer by measuring absorbance at 600 nm at intervals of 24 hours and the growth was analysed for 14 days.

Lagers with different ethanol concentrations (0 %, 2 %, 4 % and 6.6 % ABV), different bitterness range (15-28 IBU) were selected to evaluate beer spoilage ability of *Pectinatus* and *Megasphaera* isolates. The properties of the beers used in the study are shown in Table 3.4. The putative *P. cerevisiiphilus* isolates (ICBD strains PC-1 and PC-2) were able to grow in the lagers with 0-4 % ABV while they failed to grow in lager with 6.6% ABV. Putative *P. frisingensis* isolates (ICBD strains PF-1 and PF-2) were also able to grow in the lagers with 0 to 4 % ABV with the exception of ICBD strain PF- 3 which failed to grow in lager with (4 % ABV). All the putative strains of *M. cerevisiae* (ICBD strain MC-1, MC-2 and MC-3) were able to grow in lager with 0 and 2 % ABV but failed to grow in lager with 4% and 6.6 % ABV.

The samples were incubated anaerobically for 14 days at 30ºC. The concentration of acetic acid and propionic acid in different beers inoculated with putative *Pectinatus* strain ICBD strain PC-1, PC-2, PF-1 and PF-2 was determined using HPLC (section 3.8.5.2). The flavour threshold of acetic acid in beer has been reported to be < 200 ppm. The normal range of acetic acid found in beer is found to be < 100 ppm and the flavour threshold of propionic acid is 100 ppm and normal range in beer is < 100 ppm (Meilgaard, 1974; Seibert, 1999).

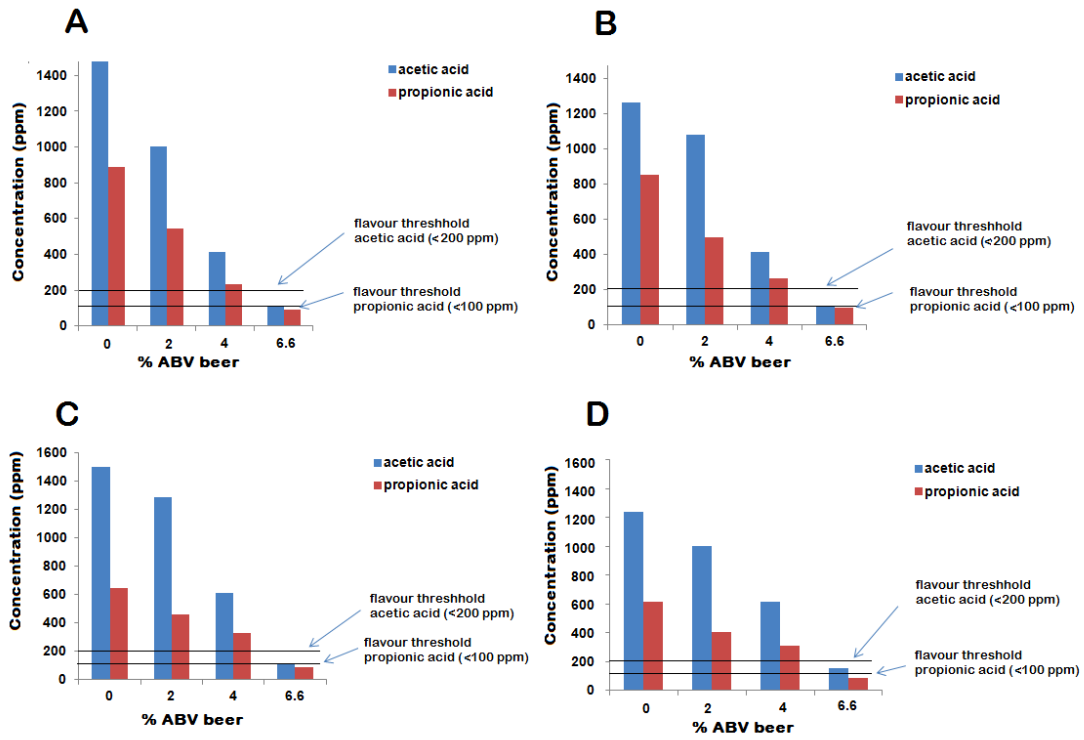


Figure 4.7 Acetic acid and propionic acid profiles for the presumed isolates of *P. cerevisiiphilu*s and *P. frisingensis* isolates. **A**, **B** represent data for ICBD strain PC-1and PC-2 respectively while **C**, **D** represent organic acid profiles for (ICBD PF-1 and PF-2 respectively. The samples were incubated anaerobically for 2 weeks at 30ºC.

Putative *P. cerevisiiphilus* isolate PC-1and PC-2 showed strong ability to spoil 0 % and 2 % ABV beer, the concentration of acetic acid and propionic acid were considerably higher than the flavour threshold of these organic acids, while the growth of isolates ICBD strain PC-1 and PC-2 in beer with 6.6 % ABV was completely inhibited . The presumed *P. frisingensis* isolate (ICBD strain PF-1and PF-2 showed strong ability to spoil 0%, 2 % and 4 % ABV beers, producing acetic acid and propionic acid higher than the flavour threshold values of these organic acids. Growth of the isolate PF-1 and PF-2 in beer at 6.6 % ABV was inhibited. The acetic acid and

propionic acid profiles for presumed *Pectinatus* isolates is shown in the Figure 4.7 The organic acid profile for presumed isolates of *M. cerevisiae* was not determined.

4.12 Genetic characterisation

Partial 16 S ribosomal DNA gene sequencing was performed to identify the presumed species of *Pectinatus* and *Megasphaera*. The nucleotide BLAST search identified ICBD strains PC-1 and PC-2 as *P. cerevisiiphilus* strain CECT 4927 (99 % max identity score) and *Pectinatus spp. (*Strain C5) (99%) respectively. Both the ICBD strain PF-1 and PF-2 were identified as *P. frisingensis* CCM 6270 (97 %) and the PF-3 isolate was identified as *P. frisingensis* strain M 50-18 (98 %). Both the putative *M. cerevisiae* isolates ICBD MC-1 and MC-2 were identified as *M. cerevisiae* DSM 20462 (99%). ICBD strain MC-3 was assigned as *M. cerevisiae strain* R 36 (98%).

CHAPTER: 4 RESULTS

PART III

CHAPTER: 4 RESULTS

PART III Hybridisation Protection Assay

3.13 RNA isolation and stabilisation

The quality and quantity of isolated bacterial RNA is important for RNA based assays hence, a separate RNA handling area was maintained within the laboratory for this study. DEPC (0.1%) treated sterile deionised water was utilized for preparation of all the reagents to inhibit RNAse activity (Sambrook and Russell, 2001) and RNasezap® (Ambion Biotech) was used for cleaning of work benches and electrophoresis apparatus to inhibit any RNAse activity.

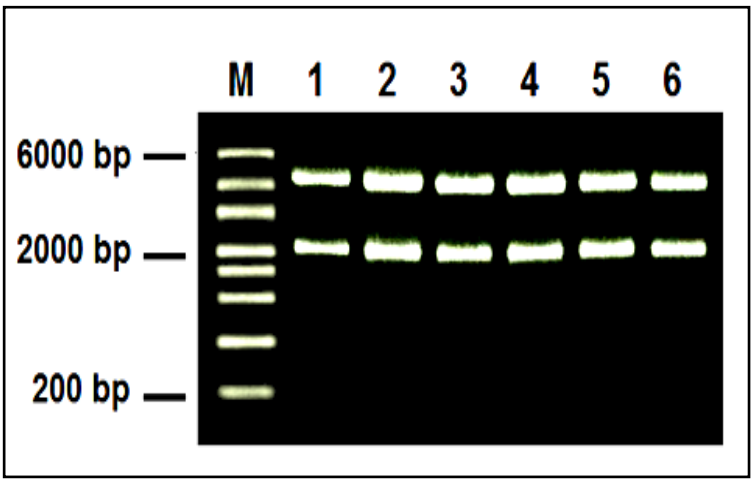


Figure 4.8 Denaturing MOPS gel electrophoresis of bacterial RNA 1) *P. cerevisiiphilus* (DSM 20467), 2) *P. frisingensis* (DSM 6306), 3) *P. haikarae* (DSM 16980), 4) *M. cerevisiae*, 5) *M. paucivorans*, 6) *M. sueceinsis*. M represents high range RNA ladder (Riborular ™ Thermo Scientific).

The quantity of RNA was measured using a spectrophotometer and quality of RNA was detected using denaturing 1% MOPS gel electrophoresis; 1 % formaldehyde was used as denaturing agent (Sambrook and Russell, 2001). Formamide (used in loading buffer preparation) denatures RNA and stabilises it during electrophoresis while formaldehyde (used in gel and loading buffer preparation) prevents formation of secondary structure in RNA (Ausubel, 1990). Denaturing MOPS gel electrophoresis of bacterial RNA is shown in the Figure. 4.8.

3.14 Probe selection and modifications

All the probes designed were based on phylogenetically conserved region of the 16S rRNA gene. A single species specific probe was developed for each of *P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae* (PC, PF and PH respectively). One genus-specific probe was also designed for all three beer spoilage *Pectinatus* (PCFH). For beer spoilage *Megasphaera* species a single probe was designed for *M. cerevisiae* (MC) while *M. paucivorans* and *M. sueceinsis* shared a single probe (MPS).

The length of the probes used for HPA typically range between 10-50 bp (Hogan, 2000). For the present study the length of the probes was 26-27 bp. A T_m value of 59-65 ºC was obtained for all of the probes. Incubation temperature of 60ºC during hybridization and differential hydrolysis is favoured in many ways relating to hybridization kinetics, stability of AE-labelled probes and specificity of reaction (Nelson *et al*., 1995). Another factor taken into consideration during the probe design was low (G+C) content (Wetmur, 1991). Probes with high G+C content have an increased T_m and also exhibits unspecific binding which could affect specificity of the probes. Similarly, repetition of the same base in consecutive sequence (4-5 times) was also avoided.

For the present study two types of AE labelled probes were developed: 5' amine modified AE probes and internal AE labelled probes using an amine modified thymidine base. The DNA probes used in this study aligned with the sequences from target species and relative bacteria is shown in Table 4.6

Table 4.6 DNA probes aligned with the sequence from target and related bacteria

	Sequences[2, 3,4]	Position[1]	Mismatch
Probe PC	3′-CTGTAACTAACTGCA**T**ACGTCTCTACG-5′		
P. cerevisiiphilus	5′-GACATTGATTGACGTATGCAGAGATGC-3′	1002-1028	0
P. frisingensis	5′-GACATTGATTGACGTATCCAGAGATGG-3′	1000-1025	1
P. haikarae	5′-GACATTGATTGACGCATTCAGAGATGG-3′	1004-1029	2
Probe PF	3′-GCGTCCGCC**T**TGTAATTCGCCTAGAA-5′		
P. cerevisiiphilus	5′-CGCAGGCGGATGACTAAGCGGATCTT-3′	587-612	3
P. frisingensis	5′-CGCAGGCGGAACATTAAGCGGATCTT-3′	587-621	0
P. haikarae	5′-CGCAGGCGGACATTTAAGCGGATCTT-3′	591-616	3
Probe PH	3′-AGGCTTGAC**T**TACAGAACTCACGTCC-5′		
P. cerevisiiphilus	5′-TTCGAACTGGTCATCTTGAGTGCAGG-3′	643-668	3
P. frisingensis	5,-TCCGAACTGAGGTTCTTGAGTGCAGG-3′	643-668	2
P. haikarae	5′-TCCGAACTGAATGTCTTGAGTGCAGG-3′	647-672	0
Probe PCFH	3′-CAGAC**T**AATCGATCAACCACTGCCATT-5′		
P. cerevisiiphilus	5′-GTCTGATTAGCTAGTTGGTGACGGTAA-3′	243-269	0
P. frisingensis	5′-GTCTGATTAGCTAGTTGGTGACGGTAA-3′	243-269	0
P. haikarae	5′-GTCTGATTAGCTAGTTGGTGACGGTAA-3′	245-271	0
Probe MC	3′-AACTCACGG**T**CCCTATCTCTATAGGAC-5′		
M. cerevisiae	5′-TTGAGTGCCAGGGATAGAGATATCCTG-3′	--	0
M. paucivorans	5′-CTACGTGCCAGCAGCCGCGGTAATACG-3′	435-462	>3
M. sueciensis	5′-CTACGTGCCAGCAGCCGCGGTAATACG-3′	435-462	>3
Probe MPS	3′-ACTGTAACTAAC**T**TGCTCCGTCTCTA-5′		
M. cerevisiae	5′-TGACCTTTAGAAGAAAAGATGGCCAC-3′	--	>3
M. paucivorans	5′-TGACATTGATTGAACGAGGCAGAGAT-3′	1090-1044	0
M. sueceinsis	5′-TGACATTGATTGAACGAGGCAGAGAT-3′	991-1006	0

1. Positions of sequences within the 16S ribosomal gene are provided based on NCBI BLAST alignments when compared to the culture collection strains, *Pectinatus cerevisiiphilus* (ATCC29359); *Pectinatus frisingensis* (ATCC33332); *Pectinatus haikarae* (VTT E-88329); *Megasphaera cerevisiae* (VTT-E-85230); *Megasphaera paucivorans* (VTT E-032341); and *Megasphaera sueciensis* (VTT E-97791).
2. The probe sequences are shown in (3'to 5') directions, whereas the 16S ribosomal sequences are shown in (5' to 3') direction
3. The position of mis-match bases 16S ribosomal gene of related organisms when compared to the target is **highlighted**
4. The thymidine base (T) position used for replacement with internal amine modified thymidine base is shown in **BOLD** letters

3.14.1 5’ amine modified AE probe

An acridinium ester molecule was attached to a DNA oligonucleotide probe using a 5’ amine linker arm. Amine linker arms of variable lengths are available; for the present study a C6 amine linker arm was used. The linker arm is attached to the 5’ end via a single ethylene- phosphate bond (Nelson *et al*., 1996) and the other end is linked to a propionate linker arm of an AE molecule via an amine group. Oligonucleotide probes with or without AE molecules show negligible difference in T_m. Hence the amine linker arm causes minimal structural defects and compatible with use as a hybridization probe for HPA assay (Nelson *et al*., 1996). The structure of an AE molecule attached to 5’ end of amine modified oligonucleotide is illustrated in Figure 4.9.

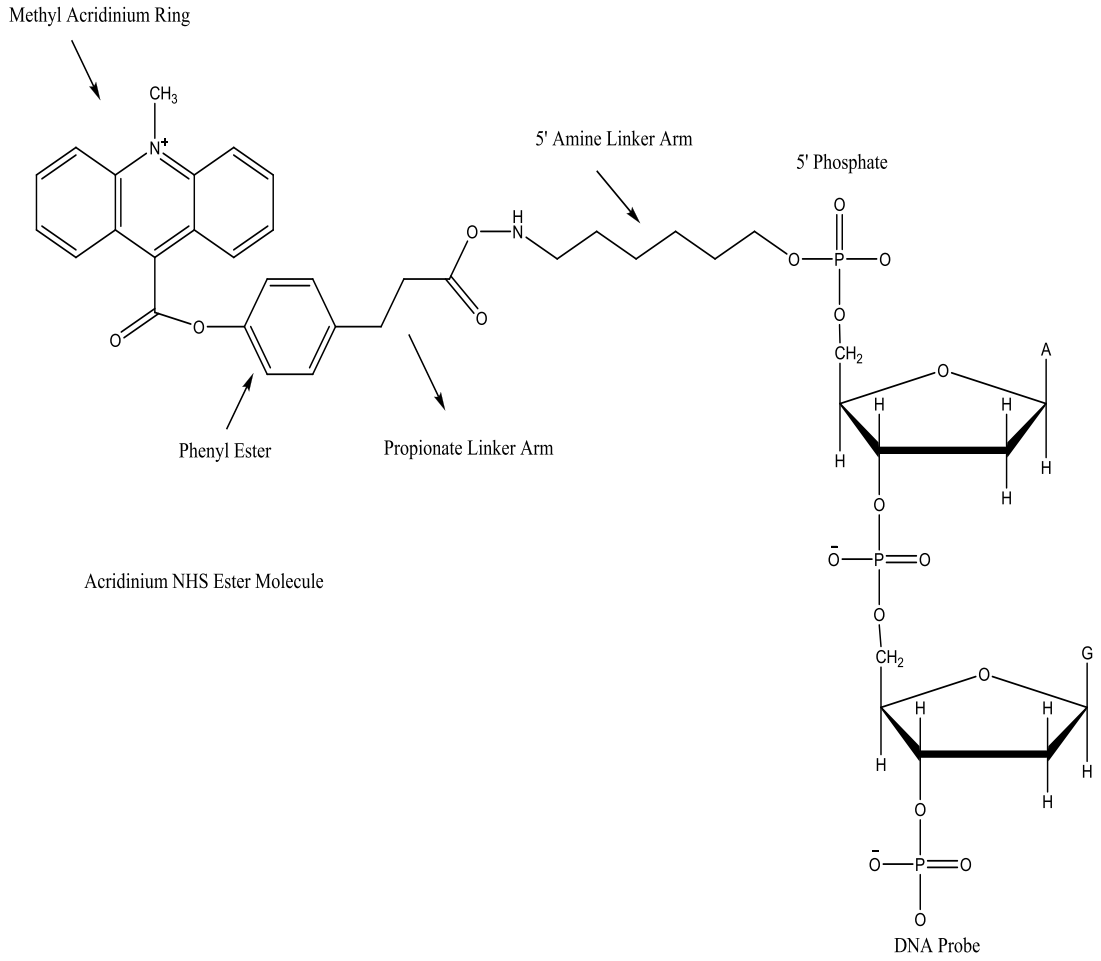


Figure 4.9 5’ end amine modified AE labelled DNA probe.

3.14.2 Internal modified AE labelled DNA probe

During the synthesis of oligonucleotides a selected thymidine base is replaced by amine-modified C6 deoxythymidine (Arnold *et al*., 1989; Nelson *et al*., 1996). The propionate linker arm of an AE-NHS ester group is covalently bound to the amine group of modified thymidine. The details of site of modified thymidine base replacement for all the probes are shown in Table 3.5 while the structure of an internally modified AE labelled probe is illustrated in Figure 4.10.

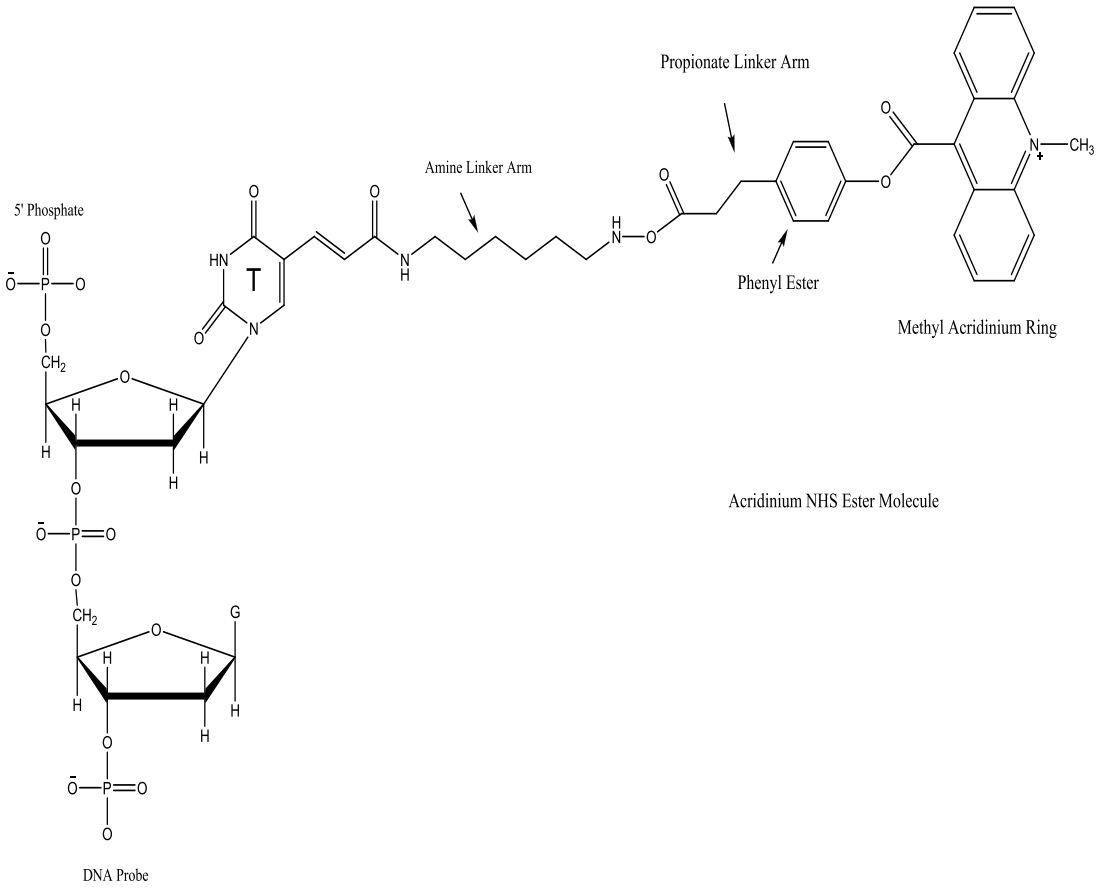


Figure 4.10 AE labelled DNA probe with internally amine modified thymidine base.

3.15 Labelling and Purification

For detection purposes it is important to obtain highly purified AE labelled probe. The presence of unlabelled probe, free AE molecules and hydrolysed AE labelled probes could significantly increase background noise, reducing sensitivity of the overall assay (Arnold and Nelson 1993, 1999)

Labelling of DNA oligonucleotide with AE has its own limitations. AE-labelled DNA probe is stable only at certain temperatures and pH. The AE molecule is incorporated at a single site in a target molecule, and hence hydrolysis of AE-labelled probe renders it inactive and unsuitable for use in HPA assay. Further, amine modified probes are difficult to label with AE as they tend to interact with negatively charged phosphate bonds of nucleic acid (Arnold and Nelson, 1993). The labelling protocol used in the present study utilized a relatively high concentration of AE molecules (10-25 mM) for labelling (10 nM of DNA probe) in organic solvent such as DMSO or DMF and 1M HEPES buffer (pH 8.0) which showed an increase in labelling efficiency of AE probes (Arnold and Nelson, 1999).

Preliminary separation of free AE molecules from labelled and unlabelled molecules was carried out using ethanol precipitation and a DyeEx column (Qiagen). Further purification of AE-labelled probes from unlabelled probes was carried out using Reverse Phase HPLC as previously described by Arnold and Nelson, 1993; Nelson *et al*., (1995) and Mazumdar *et al*. (1998).

In an original protocol described by Arnold and Nelson (1993), a binary elution system was used. For the present study a linear gradient of buffer was maintained and the concentration of acetonitrile was increased from 10 % to 40 % over the period to 30 min and a flow rate of 0.5 ml/ min was maintained. Other than HPLC several other purification protocols have been described including ion exchange chromatography (Arnold and Nelson, 1993), SEP PAK cartridge (Mazumdar *et al.*, 1998) and PAGE (Polyacrylamide gel electrophoresis).

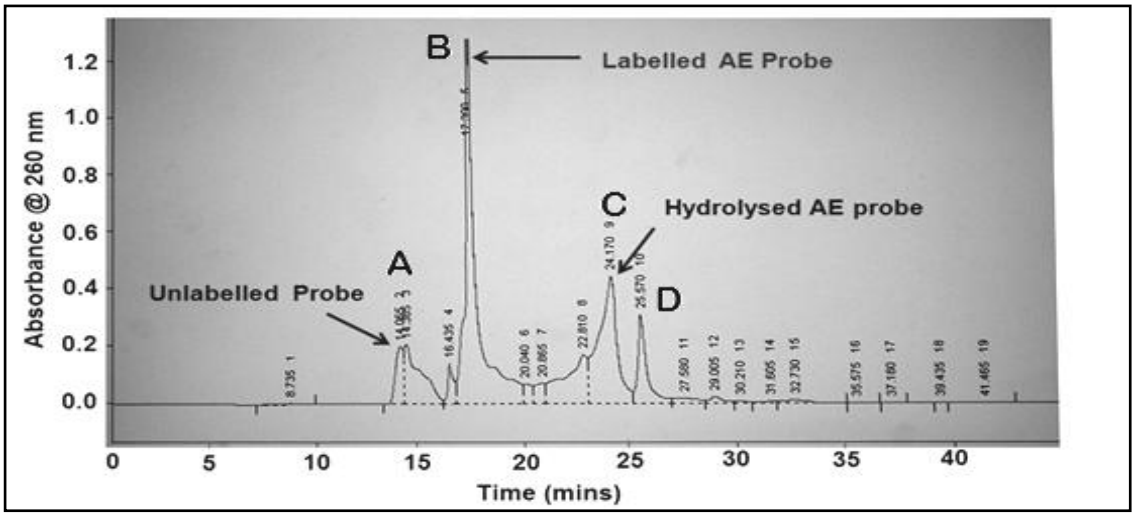


Figure 4.11 Reverse Phase HPLC purification profile for internal labelled PCFH-2 probe

All 5' end-labelled and internal AE- labelled probes followed the same pattern of elution due to the similar molecular size. A retention time for unlabelled probe was determined by eluting only unlabelled oligonucleotide through the reverse phase HPLC column. During purification unlabelled probes were eluted with a retention time of 14-15 min for end labelling probes and 14-16 min for internal labelling. Retention time for AE-labelled probes was around 17 min for both end labelled and internally labelled molecules. Finally, hydrolysed probes (probably as a result of nuclease activity) were eluted at around 24 min for end labelled probes and around 20 min for internally labelled probes. Aliquots of 0.5 ml were collected during elution and desired aliquots were pooled together and stored at -70 ºC till further use. The elution profile for internal AE-labelled PCFH-2 probe is shown in Figure 4.11.

Labelling efficiency was measured by calculating a ratio of the amount of oligonucleotide taken for labelling (pmol) to total yield of labelled AE obtained (pmol). The percent labelling efficiency for all five internal AE-labelled probes ranged from 34.6 to 59.12 %, the lowest and highest values being for PC-2 and PCFH-2 probes respectively (see Table 4.7). None of the end labelled probes achieved labelling efficiency of more than 36 %. The labelling efficiency of internal labelled probe was observed to be higher than end labelled probe of the same sequence. Labelling efficiency can be affected by various factors such as pH of labelling buffer, quality of AE stock solution, temperature and duration of labelling (Nelson *et al*., 1995). All these factors were maintained uniformly for every labelling reaction to obtain optimum labelling efficiency.

All 5' end-labelled probes are denoted as PC-1, PF-1, PH-1, PCFH-1, MC-1 and MPS-1 and all internal AE-labelled probes are referred as (PC-2, PF-2, PH-2, PCFH-2, MC-2 and MPS-2). 5' Digoxygenin labelled probes are denoted with suffix 'DIG' in the text.

Table 4.7 Labelling efficiency of DNA oligonucleotides

	Oligonucleotide concentration		
Probes	Initial amount (pmol)	Total yield (pmol)	Efficiency (%)
5' end labelled			
PC-1	10000	3601	36.01
PF-1	10000	2283	22.83
PH-1	10000	3216	32.16
PCFH-1	10000	3249	32.49
MC-1	10000	2980	29.80
MPS-1	10000	3510	35.10
Internal AE-labelled			
PC-2	10000	3460	34.6
PF-2	10000	4452	44.52
PH-2	5000	2139	42.78
PCFH-2	10000	5912	59.12
MC-2	10000	5500	55.00
MPS-2	10000	4278	42.78

3.16 Specific activity of AE probes.

Specific activity of the probes is determined by the amount of RLUs given by 1 pmol of AE-labelled probe. Insufficient labelling efficiency could result in low specific activity which can reduce the overall sensitivity of the assay. Typical range of the specific activity for AE labelled DNA probes should be around 0.5-1.0 x 10^8 RLUs/pmol (Nelson *et al.,* 1995). Slightly lower specific activity values were obtained for the all end labelled probes compared to internal AE-labelled probes of the same sequence (Table 4.8). End labelled MC-1 and MPS-1 showed the lowest values of $0.3x10^7$ and $0.35x10^7$ RLUs/pmol respectively, probably due to low labelling efficiency. For all internal labelled probes specific activity values ranged between $0.45x10^8$ and $0.9x10^8$ RLUs/pmol.

Table 4.8 Important parameters related to AE labelled DNA probes

Probes	Molecular weight[1] (g/mol)	Melting Temperature (T_m °C)	G+C content (%)	Specific activity (RLUs/pmol)
5' End labelled AE probes				
PC-1	8373	63.4	44.4	$0.28\pm.051 \times 10^8$
PF-1	8101	66.4	53.8	$0.30\pm.027 \times 10^8$
PH-1	8094	64.8	50	$0.30\pm.025 \times 10^8$
PCFH-1	8564	63.4	44.4	$0.45\pm.096 \times 10^8$
MC-1	8340	59.8	52.5	$0.30\pm.011 \times 10^7$
MPS-1	8078	61.6	42.3	$0.35\pm.022 \times 10^7$
Internal labelled AE probes				
PC-2	8348	63.4	44.4	$0.45\pm.082 \times 10^8$
PF-2	8076	66.4	53.8	$0.50\pm.026 \times 10^8$
PH-2	8069	64.8	50	$0.60\pm.034 \times 10^8$
PCFH-2	8326	63.4	44.4	$0.90\pm.043 \times 10^8$
MC-2	8333	59.8	52.5	$0.45\pm.019 \times 10^8$
MPS-2	8010	61.6	42.3	$0.45\pm.014 \times 10^8$

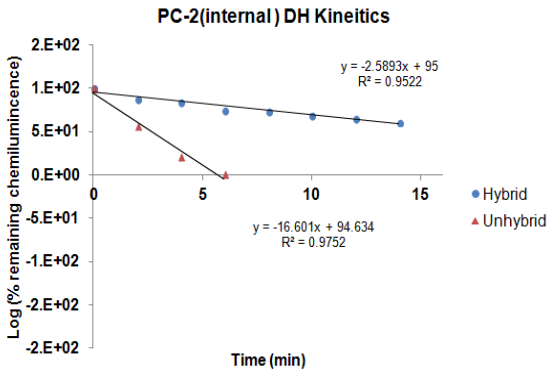
1- Difference in molecular weights of the same sequence is due to differences in molecular weights of amine linker molecules.

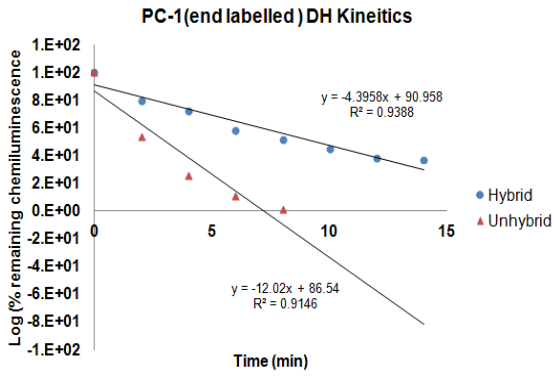
3.17 Determination of differential hydrolysis (DH) kinetics

The HPA assay is a homogenous assay which depends upon differential hydrolysis of unhybridised probes compared to hybridised probes which remain intact, therefore giving a chemiluminescence signal after reaction with alkaline peroxide (see section 2.8.2). Therefore it is important to accurately determine the optimal time for differential hydrolysis for the probes.

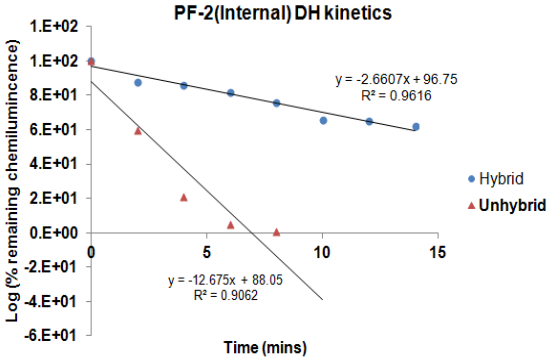
The hybridisation step was carried out at 60 ºC for 45 min to ensure optimal hybridisation and the differential hydrolysis step was carried out at pH 8.0 for 7.5 min at 60 ºC. The differential hydrolysis profiles for all the probes are shown in Figure 4.12.

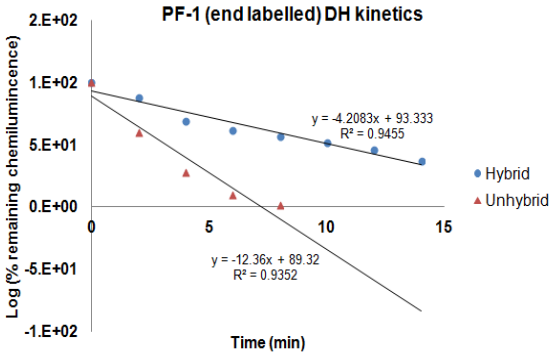
A


PC-2(internal) DH Kineitics
y = -2.5893x + 95
R² = 0.9522
y = -16.601x + 94.634
R² = 0.9752
Log (% remaining chemiluminicence)
Time (min)
Hybrid
Unhybrid

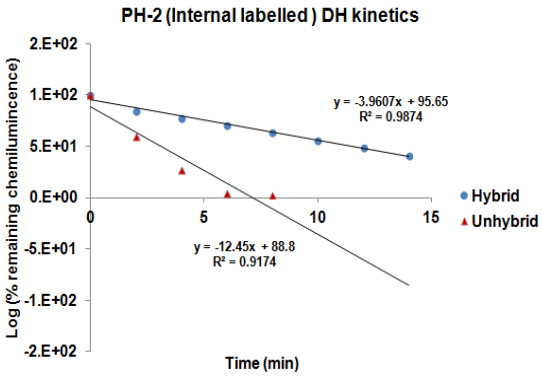

PC-1(end labelled) DH Kineitics
y = -4.3958x + 90.958
R² = 0.9388
y = -12.02x + 86.54
R² = 0.9146
Log (% remaining chemiluminescence
Time (min)
Hybrid
Unhybrid

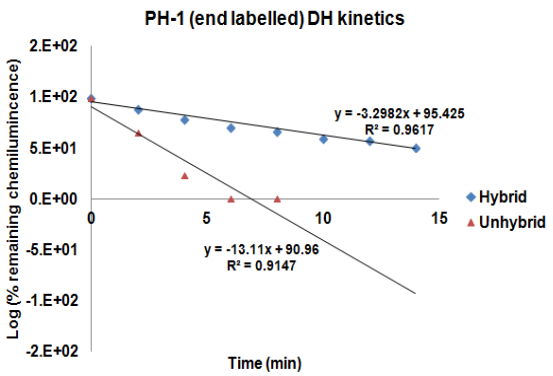
B


PF-2(Internal) DH kinetics
y = -2.6607x + 96.75
R² = 0.9616
y = -12.675x + 88.05
R² = 0.9062
Log (% remaining chemiluminicence)
Time (mins)
Hybrid
Unhybrid

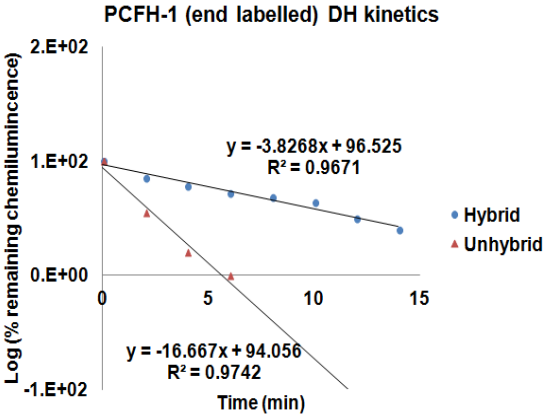

PF-1 (end labelled) DH kinetics
y = -4.2083x + 93.333
R² = 0.9455
y = -12.36x + 89.32
R² = 0.9352
Log (% remaining chemiluminicence)
Time (min)
Hybrid
Unhybrid

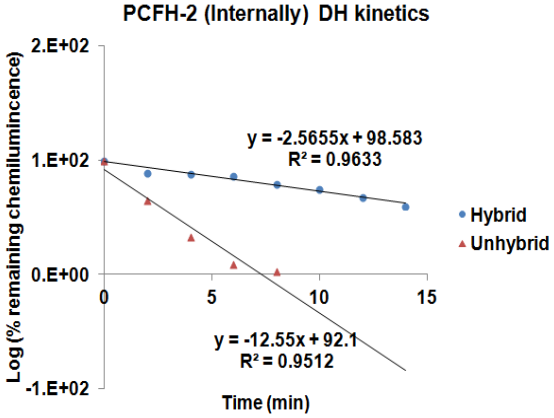
C


PH-2 (Internal labelled) DH kinetics
y = -3.9607x + 95.65
R² = 0.9874
y = -12.45x + 88.8
R² = 0.9174
Log (% remaining chemiluminicence)
Time (min)
Hybrid
Unhybrid


PH-1 (end labelled) DH kinetics
y = -3.2982x + 95.425
R² = 0.9617
y = -13.11x + 90.96
R² = 0.9147
Log (% remaining chemiluminicence)
Time (min)
Hybrid
Unhybrid

D


PCFH-1 (end labelled) DH kinetics
y = -3.8268x + 96.525
R² = 0.9671
y = -16.667x + 94.056
R² = 0.9742
Log (% remaining chemiluminicence)
Time (min)
Hybrid
Unhybrid


PCFH-2 (Internally) DH kinetics
y = -2.5655x + 98.583
R² = 0.9633
y = -12.55x + 92.1
R² = 0.9512
Log (% remaining chemiluminicence)
Time (min)
Hybrid
Unhybrid

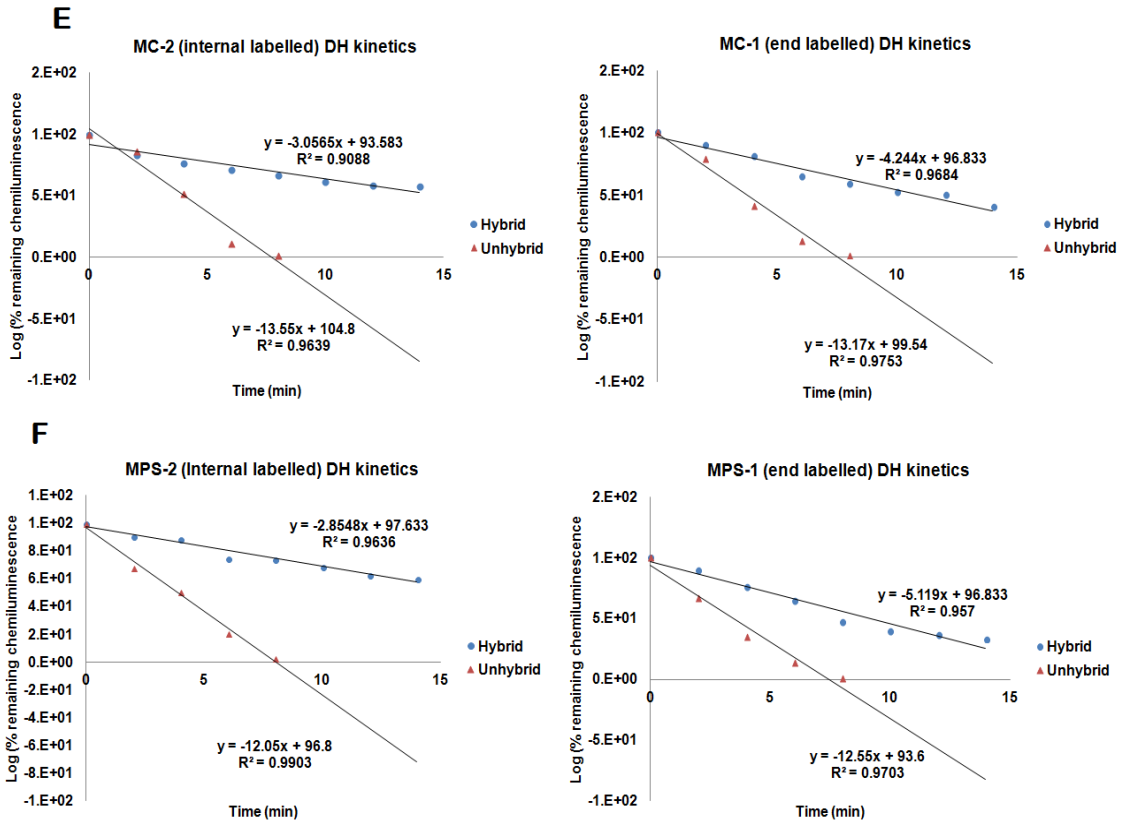


Figure 4.12 Differential hydrolysis kinetics for AE labelled probes **A, B** and **C** represent DH kinetics for internal AE-labelled and end labelled probes PC, PF and MC respectively; **D** represents DH kinetics for internal AE-labelled and end labelled probe PCFH against *P. frisingnesis* as target; **E** and **F** show DH kinetics for internal AE-labelled and end labelled probes MC and MPS respectively.

For all the probes, unhybridised sample showed a plateau stage after 8-10 min. Points beyond it were ignored for analysis because it would have caused potential errors in the determination of half lives of hybridised and unhybridised probes. For hybridised samples plateau stage was reached after 14 min hence points after 14 min were also ignored in order to accurately determine the half lives of the probes.

The DH kinetics were analysed by plotting a graph of log of percent remaining chemiluminescence (% RLUs) versus time (min), where percent chemiluminescence at time zero was taken as 100% and relative points were plotted as log (% remaining chemiluminescence). For each probe half life was determined for hybridised and unhybridised sample using standard regression analysis (Nelson *et al.*, 1995). The half lives of all the probes are shown in Table 4.9.

Table: 4.9 Half lives of AE-labelled DNA probes derived from differential hydrolysis

Probes	Half life (min)*			
	Internal AE-labelled		End Labelled	
	Hybridised	Unhybridised	Hybridised	Unhybridised
PC	19.63	1.18	9.76	1.04
PF	20.30	1.13	9.76	1.29
PH	10.88	1.56	14.84	1.25
PCFH	18.99	1.46	12.49	0.90
MC	17.81	1.40	10.59	1.27
MPS	19.12	1.56	8.75	1.20

* values are expressed as half life (min)

For all the internal AE-labelled probes half lives for hybridised sample ranged between 17.81-20.30 min and for unhybridised samples 1.13-1.56 mins. Relative half lives of hybrid samples of internal AE-labelled probes were found to be higher than those of end labelled AE probes of the same sequence and half lives for the non-hybrid samples showed no noticeable changes. The performance (labelling efficiency, specific activity and DH kinetics) of end labelled AE probes was comparatively inferior when compared to the performance of internally labelled AE probes of the same sequence.

3.18 Optimisation of HPA Assay

All optimisation experiments were carried out using internal AE-labelled PCFH-2 and PC-2 probes. The analysis of effect of time of DH and pH of DH buffer was carried out for all the internal labelled AE probes. HPA assays were carried out using the HPA protocol described in section 3.16.

3.18.1 Effect of volume of hybridisation buffer

The volume of hybridisation buffer was varied between 50-150 µl, with all other parameters kept unchanged. The data was expressed as signal to noise ratio (S/N: a ratio of hybridised probes to the background). For internal AE-labelled PCFH-2 probe relative difference between RLU values of hybridised and unhybridised samples for the volumes 50 and 75 µl was higher (S/N = 18.76 and 17.17 respectively) compared to 100, 125 and 150 µl (S/N 13.3, 13.07 and 5.8 respectively) (Figure 4.13). Above 75 µl, relative differences between RLU values of hybridised and unhybridised samples decreased gradually. This could be because a higher volume of hybridisation buffer results in insufficient mixing of DH buffer resulting into higher background noise. No noticeable difference was observed as RLU obtained at 50 and 75 µl. Similar results

were obtained for internal AE-labelled PC-2 probe. S/N ratio at volumes of 50 and 75 µl was found was found to be 16.22 and 13.23 respectively.

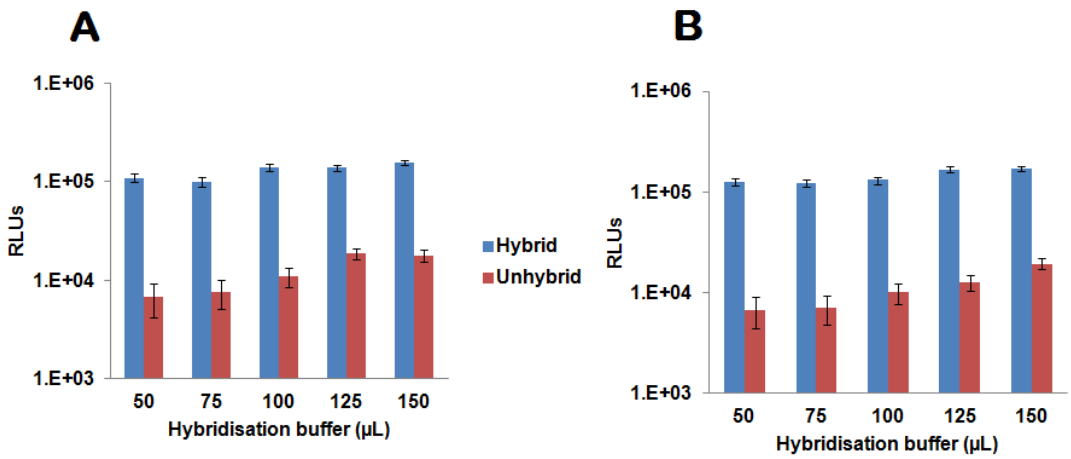


Figure 4.13 Effect of volume of hybridisation buffer on chemiluminescence performance of internally labelled AE probes. **A** and **B** represent data for internal AE-labelled probes PCFH-2 and PC-2 respectively. Error bar represents SD where (n=5).

3.18.2 Effect of time of hybridisation

For internal AE- labelled PC-2 and PCFH-2, no noticeable differences were found in RLU values obtained for hybridisation time between 30-60 min. For PCFH-2 probe S/N values obtained for 30, 45 and 60 min were 13.72, 16.22 and 16.06 respectively. In a similar way, with the PC-2 probe S/N values obtained for 30, 45 and 60 min were 14.76, 18.71 and 17.44 respectively. The lowest S/N ratio was obtained for 15 min hybridisation time, being 6.18 and 7.3 for internal AE-labelled probes PCFH-2 and PC-2 respectively. The details are shown in Figure 4.14.

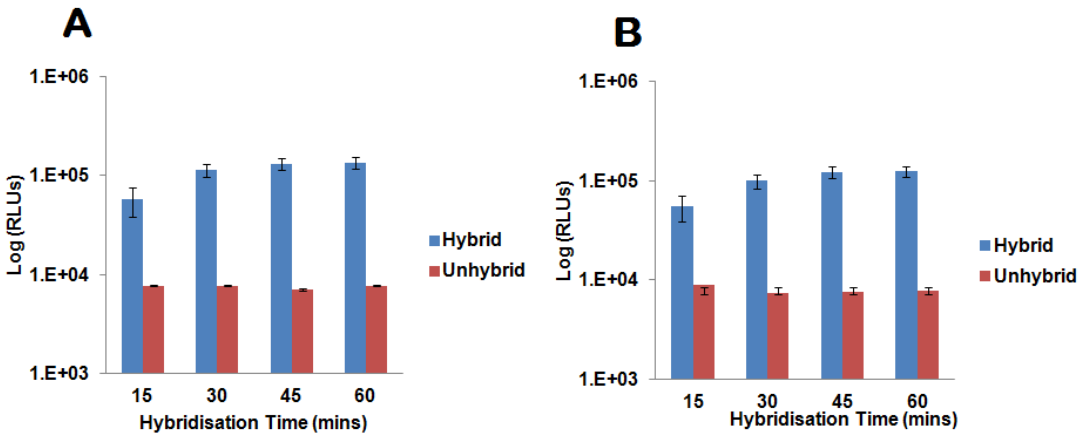


Figure 4.14 Effect of hybridisation time on performance of internally labelled AE probe **A** and **B** represents log (RLUs) values for AE probes PCFH-2 and PC-2 probe respectively. Error bar represent SD (where n=5).

3.18.3 Effect of pH and time of differential hydrolysis (DH)

The pH and the time of DH are two important parameters for HPA assay; hence performance of all the internal AE- labelled probes were evaluated at different pH and time values of DH. Effect of pH on the chemiluminescence signal obtained from all the internal AE-labelled probes is shown in Table 4.10

The pH of DH ranged from 7.0-9.0 at an interval of 0.5 units and samples were analysed with a DH time of 5, 7.5 and 10 min. The data are represented as signal to noise ratio (S/N) ratio of chemiluminescence (RLUs) for hybridised sample to that of unhybridised sample at the given time. At pH 7 and 7.5 the S/N values for DH time 5 and 7.5 min were generally lower compared to S/N value obtained at DH time 10 min (except probe PF-2). At pH 7.0 and 7.5 the strength of DH buffer was apparently too weak to allow efficient hydrolysis of unhybridised probes hence high RLU values were obtained for unhybridised samples at DH time 5 and 7.5 min, resulting in a low S/N ratio. In contrast at pH 8.5 and 9.0, the strength of the DH buffer was too high which caused hydrolysis of even hybridised probes to some extent at time 7.5 min and above, giving low RLUs values.

Table 4.10 Effect of different pH on chemiluminescence of internal AE-labelled probes

Probe	Time (min)	pH[1,2,3]				
		7	**7.5**	**8**	**8.5**	**9**
PC-2	5	03.42±.19	6.58±.38	04.27±.72	07.8±.33	**14.88±1.8**
	7.5	04.40±.20	8.70±.14	24.84±.78	**15.66±1.2**	02.92±.28
	10	**13.65±.99**	**21.8±1.1**	**30.36**±1.3	24.50±1.4	02.20±.29
PF-2						
	5	07.80±.19	11.23±1.0	08.74±.67	**24.11**±1.3	**20.12**±1.1
	7.5	**27.45**±.86	21.61±1.5	**30.13**±1.3	22.64±.73	06.41±.67
	10	25.29±1.9	**22.34**±1.0	25.88±.66	14.17±.81	03.44±.13
PH-2						
	5	05.89±.33	06.9±.34	13.96±.76	**14.56±.84**	**16.22±.62**
	7.5	07.66±.45	10.06±.89	**20.99±95**	08.73±.61	04.94±.77
	10	**17.33±.93**	**19.9±.78**	20.10±1.1	02.30±.16	02.39±.14
PCFH-2*						
	5	06.10±.44	09.2±.83	10.10±.98	**18.34±.84**	**21.44±1.6**
	7.5	18.21±1.3	15.97±1.1	**28.57±1.1**	12.66±.71	05.92±.94
	10	**18.95±.76**	**20.58±1.5**	21.87±.98	08.80±.92	03.18±.75
MC-2						
	5	4.57±.31	7.7±.54	4.11±.69	**21.80±.61**	**19.5±.1.5**
	7.5	7.76±.62	18±.56	**30.54**±.83	14.89±.88	5.92±.32
	10	**11.67**±.97	**17.34±.93**	27.78±.78	17.56±1.0	04.2±.91
MPS-2						
	5	05.20±.99	**05.6±.41**	12.03±.81	**18.90±.89**	**20.45±1.2**
	7.5	05.73±.45	**18.93±.73**	**22.23±.82**	15.45±.90	12.65±.67
	10	**18.11±.87**	18.55±.94	20.24±1.2	10.30±1.0	07.30±.83

1. The data are represented as signal to noise ratio (S/N) obtained HPA assay using internal AE labelled probes
2. The values are expressed as mean S/N ratio of 5 replicates.
3. Optimum S/N values within the given parameters are highlighted in **BOLD** letters

* The values for PCFH-2 probes are shown using RNA obtained from *P. frisingensis* (DSM 6306)

At pH 8, S/N ratio at time 7.5 min was found to be highest for all the internal AE-labelled probes (except PC-2). For all the probes S/N values at 10 min were lower compared to 7.5 min except PC-2 probe which showed highest S/N 30.36 at the given time. It could be concluded that for all the internal AE- labelled probes the optimal time for DH at pH 8.0 lies between 7.5 mins and 10 mins. It could be possible to obtain optimal DH condition using higher pH and shorter DH time. However the rate of hydrolysis is much higher at high pH (8.5, 9.0), hence small errors in sample handling could give incorrect results (Nelson *et al.,* 1995), and hence DH at pH 8.0 for time 7.5-

10 min was used for this study. The summary of optimised HPA conditions used for this study is shown in the Table 4.11.

Table 4.11 Hybridisation and DH conditions for AE labelled probes

Hybridisation	
Buffer	50- 75 µL of succinate buffer 1 (0.1 M lithium succinate (pH 5.2), 2 mM EDTA, 2mM EGTA, 10 % lithium dodecyl sulphate (w/v)), 25 fmol of AE labelled DNA probe and 1 pmol of target nucleic acid.
Conditions	Temperature : 60 ºC; Time: 45 min; Volume : 50- 75 µL.
Differential hydrolysis	
Buffer	300 µl of 0.125 M sodium tetraborate (pH 8.0), 5 % Triton X-100
Conditions	Temperature : 60 ºC; Time: 7.5-10 min; Volume : 300 µL.
Detection	
Detection	200 µl of 0.4 M HNO_3, 0.1 % H_2O_2 and 200 µl of 1 M NaOH
Time	5 sec using dual injector luminometer (Berthold model- LB-9706)

3.19 Specificity of acridinium ester probes

The specificity of AE probes was determined by using low stringency hybridisation buffer. Low stringency hybridisation buffer containing 0.4 M lithium succinate as a source of monovalent cations was used since monovalent cations tend to stabilise the DNA-RNA hybrid (Wetmur, 1991). The specificity of AE labelled probes was analysed against closely related bacterial RNA sample along with some other bacteria (Figure 4.15 and 4.16). The RNA sample (excluding the target RNA) giving maximum mean RLUs was taken as cut- off value (optimum statistical RLU values obtained for unhybridised sample); above which a sample was considered to be positive for bacterial detection.

The target sequence of PC-2 probe on the 16S RNA gene of *P. cerevisiiphilus* includes 1 and 2 mismatched nucleotides when compared to the sequences of type strains of closely related beer spoilage bacteria; *Pectinatus frisingensis* (DSM 6306) and *P. haikarae* (DSM 16980) respectively (Table 3.7). It was significant to report that internal AE- labelled PC-2 probe could clearly distinguish between *P. cerevisiiphilus* and *P. frisingnesis*. The S/N ratio for *P. cerevisiiphilus* and *P. frisingensis* was 17.17 and 1.0 respectively. The internal AE-labelled probes PF-2 and PH-2 also showed species specific detection with S/N ratio of 25.99 and 15.47 for *P. frisingensis* and *P.*

haikarae respectively. The beer spoilage *Pectinatu*s genus specific probe PCFH-2 (internal AE-labelled) could successfully distinguish between the target species and other closely related microorganisms. PCFH-2 probe gave S/N ratio of 17.57, 15.01 and 14.35 with the target species *P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae* respectively. The specificity of internal AE- labelled probes PC-2, PF-2, PH-2 and PCFH-2 is shown in Figure 4.15

Internal AE-labelled MC-2 probe was also able to detect target RNA with S/N ratio of 12.6. The internal AE-labelled MPS-2 probe gave the lowest S/N ratio among all probes, the S/N ratio obtained for *M. paucivorans* and *M. sueceinsis* being 10.45 and 10.29 respectively. *M. cerevisiae* gave relatively higher RLU values for the probe MPS-2 hence the cut-off value was relatively high thus lowering the S/N ratio to some extent. An S/N ratio of >10 is still a relatively large difference which could be used to distinguish between hybridised and unhybridised sample. Specificity of AE probes was also checked against brewery isolates, *S. cerevisiae* (production strains) and wild yeasts. All the probes could specifically detect only target bacterial RNA with high (>12) S/N ratios (data not shown).

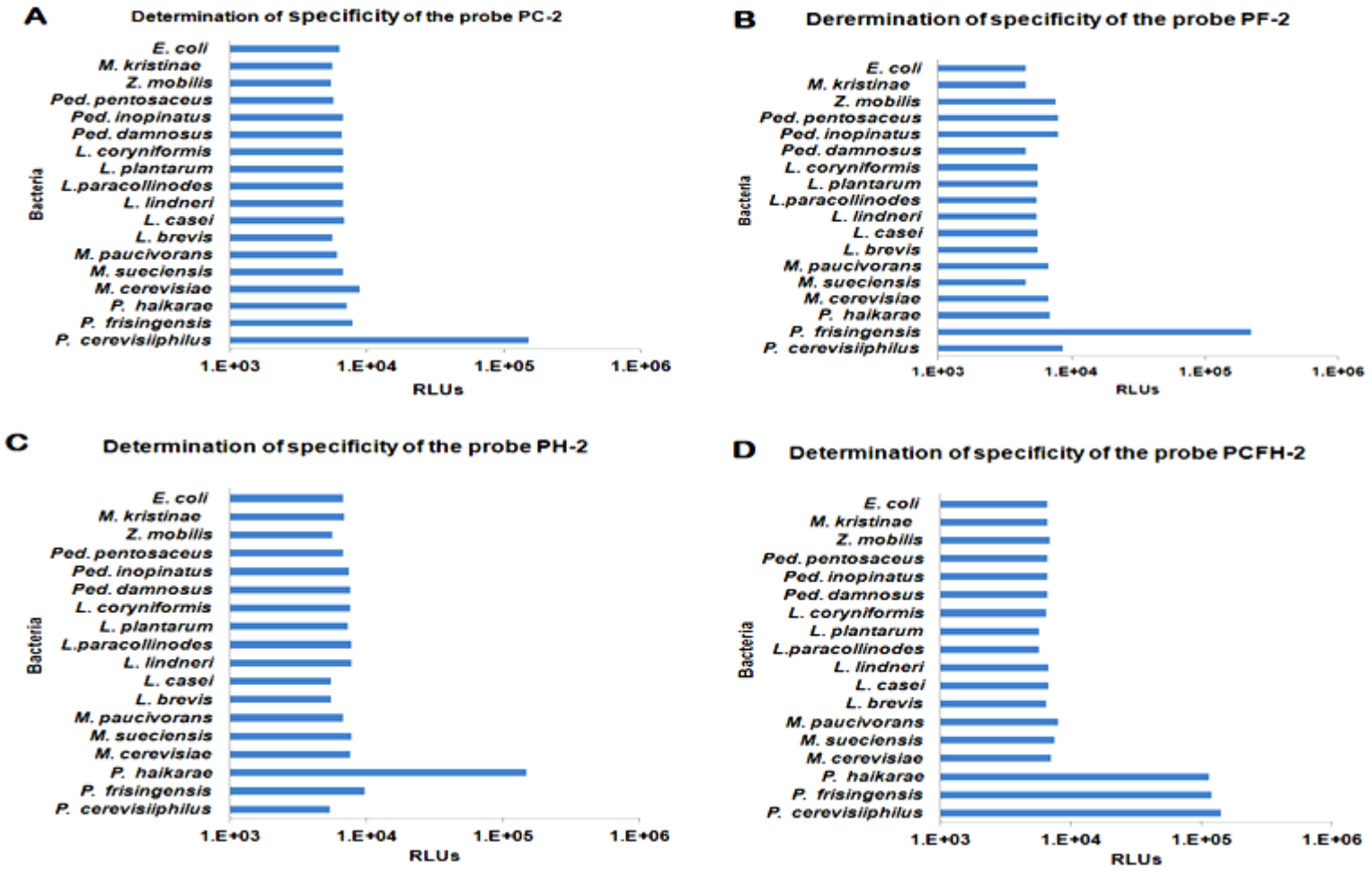


Figure 4.15 Specificity of the AE labelled probes (PC-2, PF-2, PH-2 and PCFH-2) compared to related beer spoilage microorganisms

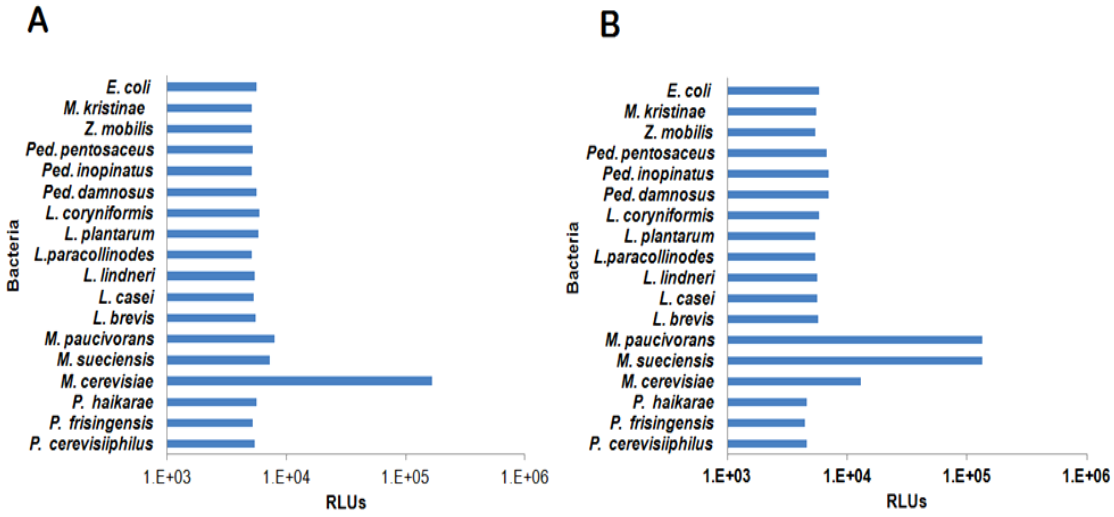


Figure 4.16 Specificity of the internal AE-labelled probes (MC-2 and MPS2) compared to related beer spoilage microorganisms

3.20 Sensitivity of AE labelled DNA probes

Total bacterial RNA concentration was measured by monitoring absorbance at 260 and 280 nm (section 3.10.4). The total bacterial RNA was assumed to be 4500-5000 bases and the concentration of RNA in pmol/ µL was calculated. The samples were five-fold serially diluted and used for HPA assay and RNA slot blot analysis. For RNA slot blot analysis the same probes as shown in Table 2.4 were used, except that the 5' end of the probes was labelled with digoxygenin.

With one exception, all internal AE-labelled probes could clearly distinguish 0.016 pmol of target RNA with S/N ratios of > 2; i.e. the RLU signal for the hybrid gave double the cut-off signal. The MPS-2 probe gave an S/N ratio of 1.40 for 0.016 pmol of the target. For 0.0032 pmol of target RNA, the PC-2, PF-2 and MC-2 probes could detect target RNA with S/N ratio of 2.14, 2.29 and 2.1 respectively. PH-2 and MPS-2 probes could not detect a target RNA amount of 0.0032 pmol as RLU values for hybridised samples were within the range of the cut off value i.e. S/N ratio of 1.01 and 1.04 respectively.

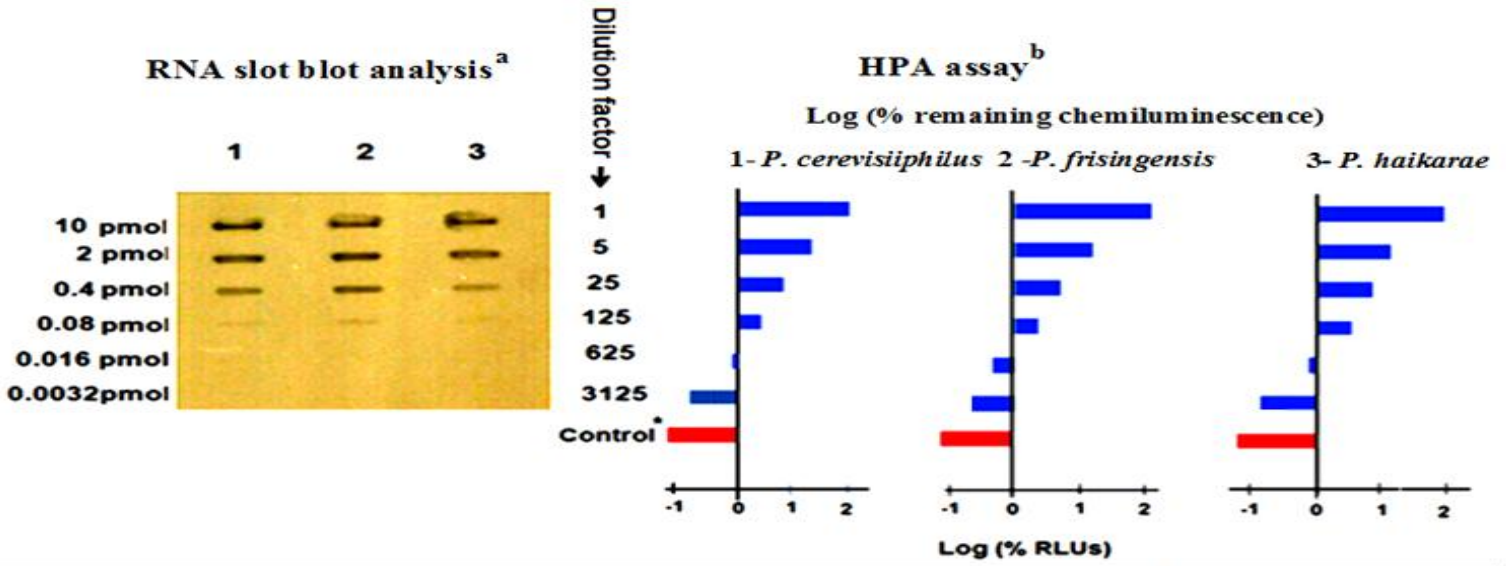
The *Pectinatus* genus specific probe PCFH-2, clearly detected 0.0032 pmol of RNA isolated from *P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae* with S/N ratio of 3.28, 2.37 and 2.22 respectively. A detection limit of 10^{-3} and 10^{-4} µg of RNA in buffer and clinical samples respectively has been described previously (Arnold and Nelson, 1999). For the present study internal AE-labelled PC-2, PF-2 and PCFH-2 probes were

able to detect ca. $1.6x10^{-3}$ µg of target RNA (1 µg = 0.67 pmol). The developed HPA assay shows similar sensitivity, compared to previous literature values (Arnold and Nelson, 1999).

A comparison between RNA slot blot analysis and HPA assay using PCFH-DIG and PCFH-2 is shown in Figure 4.17. AE-labelled PCFH-2 showed higher sensitivity of detection (0.016-0.0032 pmol) compared to the sensitivity obtained for PCFH-DIG (0.4-0.08 pmol). The time required for performing HPA assay was less than one hour compared to 8-10 hours for RNA slot blot analysis using PCFH-DIG probe. The sensitivity and specificity of the developed HPA assay was found to be better than the adapted RNA slot blot analysis.

All the DIG labelled probes were able to detect down to 0.4 to 0.08 pmol of target RNA, The DIG labelled PCFH-DIG, PH-DIG and MPS-DIG probes gave less intense signal for 0.08 pmol of target RNA compared to DIG labelled PC-DIG, PF-DIG and MC-DIG which gave clear detection signals at this concentration. None of the DIG labelled probes could detect target RNA at 0.016 pmol or below.

The specificity and sensitivity of all the DIG labelled probes was found to be satisfactory except for the PC-DIG probe which gave lower intensity signals for high amounts of RNA (2.0 and 0.4 pmol) isolated from *P. frisingensis* even after increasing the time of high stringency washing by 10 min (see section 3.18.3). In comparison, using HPA assay for the same amount of RNA (2.0 and 0.4 pmol), internal labelled-AE probe (PC-2) was able to distinguish between *P.frisingensis* RNA with S/N ratio of 28.50 and 20.53 respectively. A comparison between RNA slot blot analysis using DIG labelled probes and HPA assay using internal AE labelled probes for *Pectinatus* and *Megasphaera* species specific probes is illustrated in Figure 4.18 and Figure 4.19 respectively.



a – RNA slot blot analysis using 5'DIG labelled PCFH-Probe
Time - 8-10 hours
Sensitivity - 0.4- 0.08 pmol of RNA
Steps involved - > 5
Specificity - very good
Hybridisation - > 6hours
Film exposure - 15-60 min

b- HPA assay using internally AE labelled PCFH-2 Probe
Time - < 2 hours
Sensitivity - 0.08- 0.0032 pmol of RNA
Steps involved - 3
Specificity - very good
Hybridisation - < 1 hours
Detection step - 5 seconds

* RNA from M. cerevisiae (DSM 20461) was used

Figure 4.17 Comparisons of sensitivity of RNA slot blot analysis and HPA assay. Serially diluted RNA was subjected to RNA slot blot analysis and HPA assay. For slot blot 5' DIG labelled PCFH-1 probe was used. For HPA assay a graph was plotted relating log of (percent remaining chemiluminescence) to amount of RNA (pmol), where RLU obtained for optimal amount (10 pmol) was taken as 100 %. 1, 2 and 3 represent data for *P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae* respectively.

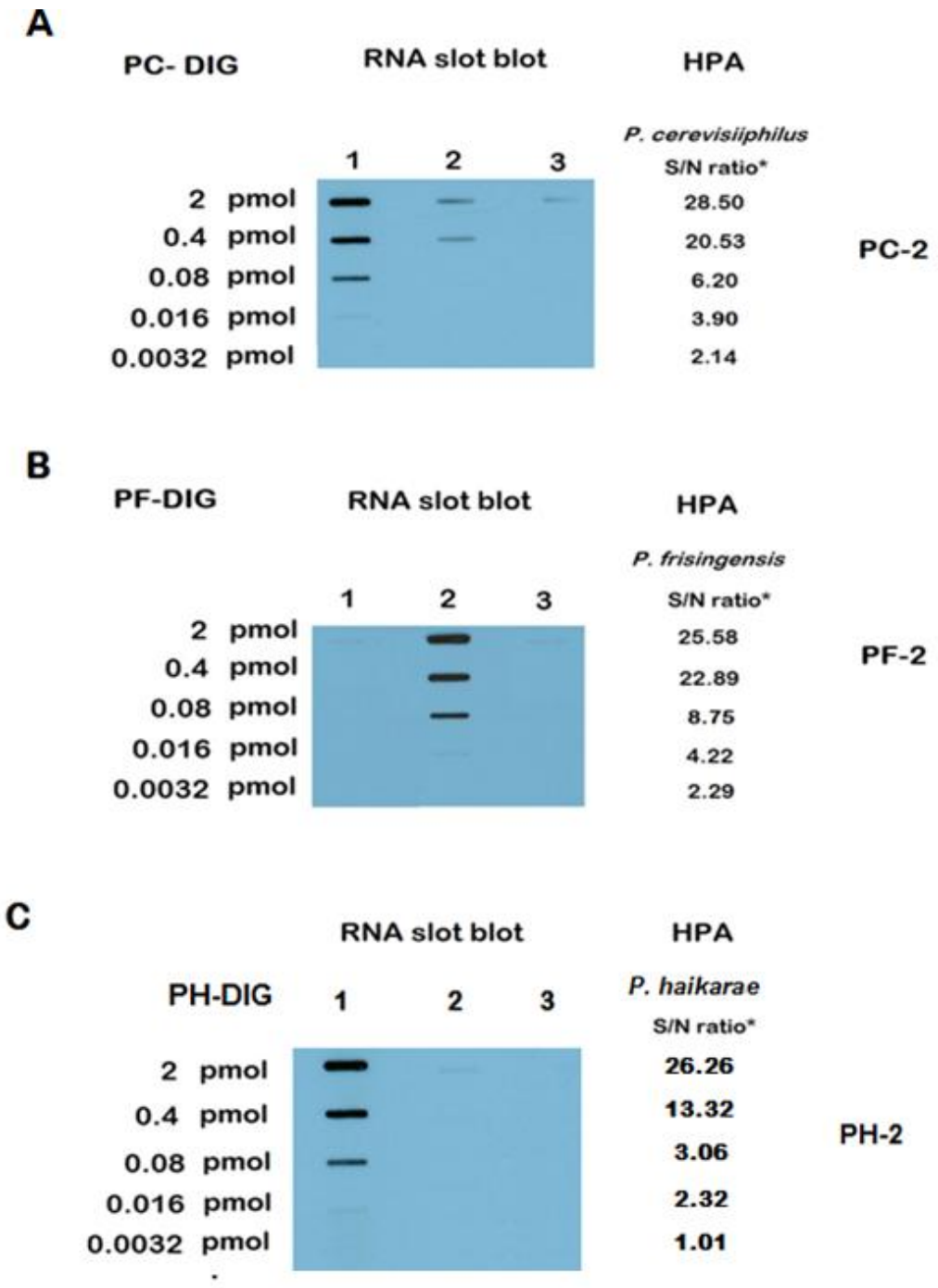


Figure 4.18 RNA slot blot analysis and HPA assay conducted for probes PC and PF and PH probes (DIG labelled and Internal AE labelled). **A** and B represent analysis of the probe PC and PF, the sensitivity of RNA slot blot analysis is limited to 0.08 pmol of target RNA while HPA assay could detect target RNA at 0.0032 pmol with S/N ratios of >2. **C** represents analysis of the probe PH, the sensitivity of RNA slot blot analysis is limited 0.08 pmol of target RNA while HPA assay could detect target RNA at 0.016 pmol with S/N ratios of >21, 2 and 3 represent serially diluted RNA from *P. cerevisiiphilus*, *P. frisingnesis* and *P. haikarae* respectively.

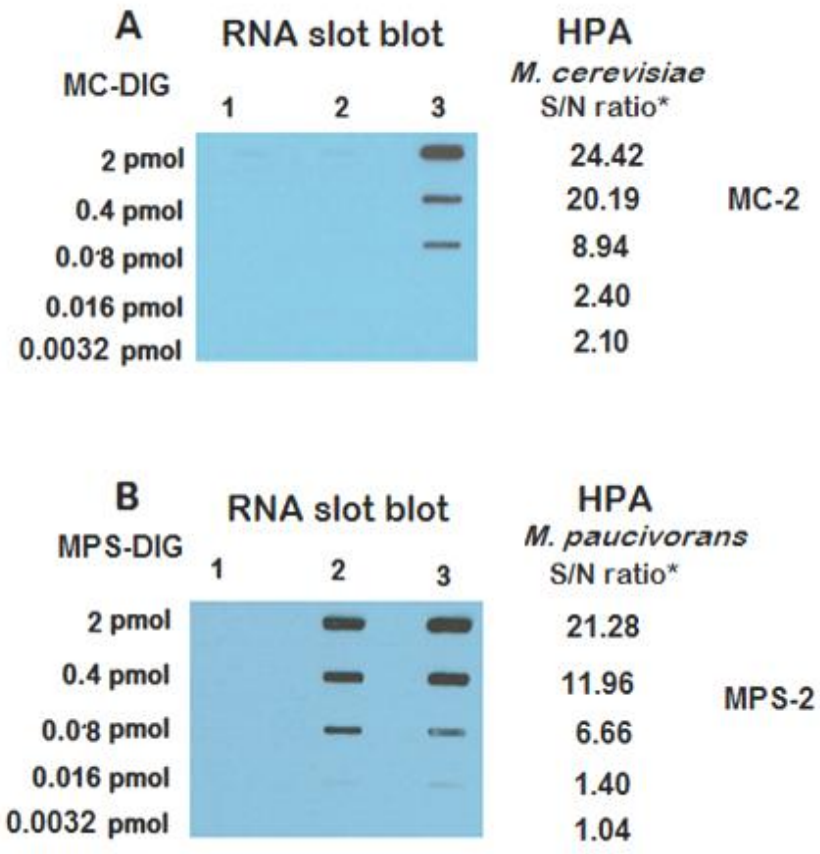


Figure 4.19 RNA slot blot analysis using DIG labelled probes. **A** and **B** represent the RNA slot blot and HPA assay result for MC and MPS probes respectively. 1, 2 and 3 represents blotting results for serially diluted RNA from *M. cerevisiae*, *M. paucivorans* and *M. sueciensis* respectively.

3.21 Application in brewing

3.21.1 Detection of background noise

Intrinsic chemiluminescence of the samples could reduce the overall HPA assay sensitivity (Nelson *et al*., 1995); hence detection of background noise for various beers was significant. Degassed beer (5-10 µL volume) was added to 50 µL of hybridisation buffer-1 (no AE probes and target RNA) and directly used for chemiluminescence detection as described in section 3.16.3. A negative control was set up using deionised water. The commercially available beers, five commercial lagers (2- 6.6 % ABV), five dark beers (3.8- 5 % ABV) and five ales (4-6 % ABV) were checked for background noise, the results are shown in the Figure 4.20.

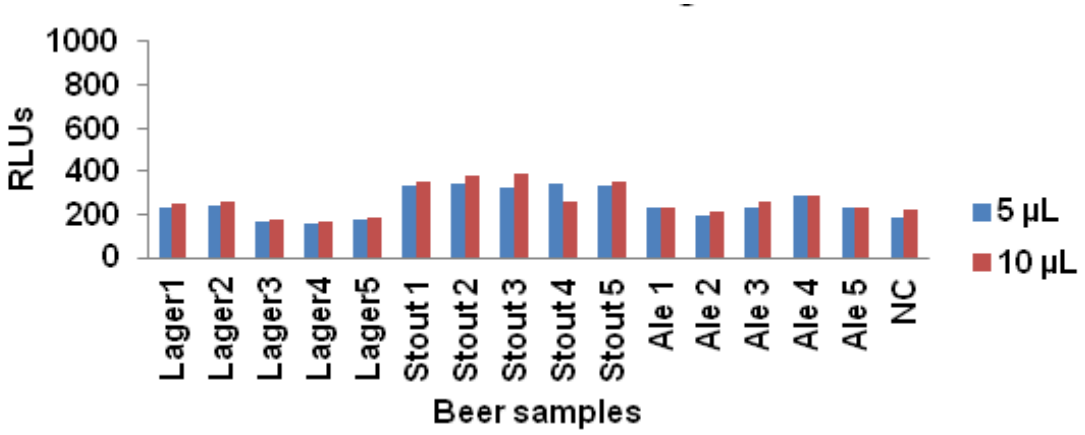


Figure 4.20 Contribution of different beers to background noise. RLUs were measured using the detection step of the HPA assay.

The commercial lager contributed least to the background noise using the detection steps used for HPA assay. For 5 and 10 µL volumes, the mean RLUs contributed by lagers were 204.8 (S.D= ±37.59) and 247.8 (S.D= ± 20.42) respectively. Dark beers showed the highest background with mean RLUs of 334 (S.D ±7.19) and 343 (S.D= ±52.66) for 5 and 10 µL volumes respectively, while the ales showed mean RLUs of 223.8 (S.D= ±32.28) and 244 ((S.D= ±30.44) for 5 and 10 µL respectively. The negative control, for 5 and 10 µL gave mean RLUs values of 190 (S.D= ±14.78) and 196 (S.D= ±7.45) respectively. The results suggested that the examined beers contributed minimally to background noise of the HPA assay especially when compared to background RLUs (approximate range 5500-9000 RLUs) obtained for hybridisation buffer containing AE-labelled probes.

3.21.2 Comparative results for cell lysis protocols

Three different cell lysis protocols were used for obtaining crude RNA ready to use for HPA assay. Effect of enzymatic lysis, detergent lysis and heat lysis protocols were performed using fresh bacterial samples and frozen bacterial pellets. The effect of RNA stabilisation on HPA assay using of fresh and frozen pellets was also studied.

Frozen pellets and fresh bacterial samples obtained from *P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae* were subjected to three different cell lysis treatments and used for HPA assay as described in section 3.21. A negative control was set up using crude bacterial lysate obtained from *M. cerevisiae* (DSM 20461) treated in a similar way as other target bacteria. The results are shown in Table 4.12.

Table: 4.12 Effect of bacterial lysis treatment on HPA assay using PCFH-2 probe

Bacteria	Lysis method	Frozen bacterial pellet		Fresh Bacterial pellet	
		S/N ratio			
P. cerevisiiphilus		**Frozen**	**BS treated[4]**	**Fresh**	**BS treated[4]**
	Enzymatic [1]	15.66*	17.58	17.77	18.76
	Detergent [2]	17.30*	18.02	18.77	20.98
	Heat lysis[3]	17.50*	18.38	18.67	19.50
P.frisingensis					
	Enzymatic[1]	15.30*	15.87	17.65	18.55
	Detergent [2]	17.50*	18.81	19.10	20.05
	Heat lysis[3]	17.90*	19.18	18.57	19.90
P. haikarae					
	Enzymatic[1]	17.79*	18.56	19.09	20.10
	Detergent [2]	17.90*	19.66	20.85	21.10
	Heat lysis[3]	18.10*	20.00	20.52	21.40

The data are represented as signal to noise ratio obtained in the HPA assay using internal AE labelled PCFH-2 probe. * Significance difference (p value < 0.05) compared to S/N values obtained fresh bacterial pellet.

1- lysozyme (15 mg/ml) and 20 µL Proteinase K (Qiagen 20 mg/ml) in TE buffer (pH-8.0)

2- cell lysis buffer (Qiagen) + incubation at 85 ºC for 10 min

3- 0.1 % SDS + heat treatment of 95 ºC for 10 min

4- pellet treated with bacterial stabilisation reagent (Qiagen) for RNA stabilisation

The data obtained for different cell treatments (fresh pellet, RNA stabilised fresh pellet, frozen pellet and RNA stabilised frozen pellet) in the form of S/N ratio was compared for statistical significance. One way ANOVA followed by Dunnet' tests, was used and the data for fresh bacterial pellet were used as control. For all three *Pectinatus* species no significance difference was noted (p value > 0.05), for S/N values obtained for RNA stabilised fresh and RNA stabilised frozen pellets. The frozen, non treated pellet showed statistical difference (p value < 0.05) when compared to the control sample (fresh pellets).

No significance difference (p value >0.05) was obtained for S/N ratio values obtained for all the *Pectinatus* species using three different cell lysis protocols. The detergent lysis protocol was therefore used for cell lysis in further study using RNA stabilised frozen pellets.

3.21.3 Bacterial CFU detection limit of HPA assay

A frozen pellet obtained (as described in section 3.21) with known CFU/ml was fivefold serially diluted and 10 µl aliquot of serially diluted sample was mixed with 90 µl detergent based cell lysis buffer and incubated at 85 ºC for 10 mins, cooled on ice for 3 mins and centrifuged briefly to precipitate cell debris. A 5-10 µl aliquot of supernatant was used as crude RNA for the HPA assay.

Limit of detection of bacterial CFU was determined for *Pectinatus* and *Megasphaera* species using internal AE-labelled probes. All internal AE-labelled probes were clearly able to detect 5x 10^3 of target bacteria with S/N ratio > 5 except the PH-2 probe where S/N of 3.9 was obtained. None of the designed probes were able to detect 2.5x10^2 CFU of target bacteria. Internal AE-labelled probes PC-2, PF-2 and MC-2 were able to detect 5x 10^2 CFU of target bacteria with S/N ratio of just over 2 (2.33, 2.10 and 2.21 respectively) but PH-1 and MPS-2 probes failed to detect 5x10^2 CFU of target bacteria showing S/N ratio of 1.56 and 1.72 respectively. Both these probes were clearly able to detection 1x10^3 CFU with S/N ratio of 2.1 and 2.37 respectively.

Genus specific internal AE-labelled PCFH-2 probe was able to detect 5x 10^2 CFU of RNA stabilised frozen pellet of *P. cerevisiiphilus, P.frisingensis and P. haikarae* with S/N ratio 3.90, 2.54, 2.22 respectively. From the results obtained it can be concluded that internal AE- labelled PCFH-2, PC-2, PF-2 and MC-2 could detect 5x10^2 CFU of target bacteria while the detection limit for the probes PH-2 and MPS-2 was between 5x 10^2 and 1x10^3 CFU.

3.21.4 Sensitivity of HPA in different ABV beer and beer enriched medium

Initial experiments were carried out to determine the ability of HPA assay to specifically detect beer spoilage *Pectinatus* and *Megasphaera* species from actual beer samples. Different ABV content lagers (0%, 2%, 4% and 6.6 % ABV) were artificially spiked with a known number of bacteria to obtain final concentration of ca.10-100 CFU/ml and incubated anaerobically at 30 ºC for 5-7 days.

Similar experiments were set up to determine the effect of enrichment of beer with different media recommended for detection of *Pectinatus* and *Megasphaera*. MRS, NBB and SMMP media were used as enrichment of media. MRS (De Man *et al.,* 1960) and NBB (Kindraka, 1987) media were mixed in 1:1 ratio (v/v) while SMMP medium was used in the ratio 15% selective media: 85% beer (v/v) as recommend by Dull *et al.,* (1998).

Negative controls were set up with beer sample or beer-enriched media without bacteria. 1 ml of the incubated samples were removed at intervals of 24 hours and lysed with detergent to obtain a crude RNA lysate as described in the methods. HPA assay was carried out using the specific AE-labelled probes. For each sample, the result was regarded as positive when the sample gave S/N ratio greater than 2, and the time required to obtain a positive result was noted, the results are shown in Table 4.13 and 4.14

Table 4.13 Sensitivity of HPA in different ABV (0 % and 2 %) beer and beer enriched medium

		Incubation time required for Detection using HPA assay[1]							
		0% ABV beer + enrichment media				2% ABV beer + enrichment medium			
Bacterium	**HPA probe[6]**	**Control[2]**	**MRS[3]**	**NBBC[4]**	**SMMP[5]**	**Control[2]**	**MRS[3]**	**NBBC[4]**	**SMMP[5]**
P. cerevisiiphilus	PC-2	72	48	48	72	72	48	48	96
	PCFH-2	72	48	48	72	72	48	48	96
P. frisingensis	PF-2	48	48	48	48	72	48	48	48
	PCFH-2	48	48	48	48	72	48	48	48
P. haikarae	PH-2	96	48	72	72	96	48	72	72
	PCFH-2	96	48	72	72	96	48	72	72
M. cerevisiae	MC-2	96	48	48	48	96	72	72	72
M. paucivorans	MPS-2	96	48	72	96	120	96	96	96
M. sueceinsis	MPS-2	ND[7]	ND	ND	ND	ND	ND	ND	ND

1- Incubation was carried out anaerobically at 30 ºC and time required to obtain S/N ratio of > 2.0 was recorded
2- Control sample specified beer with no enrichment medium added
3- Beer+ MRS medium were taken in 1:1 ratio (v/v)
4- Beer + NBBC medium were taken in 1:1 ratio (v/v)
5- (85% SMMP media component: 15 % beer)
6- Internally labelled AE probes were used for detection.
7- ND Not detected

Table 4.14 Sensitivity of HPA in different ABV (4 % and 6.6 %) beer and beer enriched medium

		Incubation time required for Detection using HPA assay							
		4% beer ABV + enrichment medium				6.6% ABV beer + enrichment medium			
Bacterium	**HPA probe[6]**	**Control[2]**	**MRS[3]**	**NBBC[4]**	**SMMP[5]**	**Control[2]**	**MRS[3]**	**NBBC[4]**	**SMMP[5]**
P. cerevisiiphilus	PC-2	72	48	48	96	N	96	72	N
	PCFH-2	72	48	48	96	N	96	72	N
P. frisingensis	PF-2	72	48	48	72	N	48	48	N
	PCFH-2	72	48	48	72	N	48	48	N
P. haikarae	PH-2	120	48	48	N	N	72	72	N
	PCFH-2	120	48	48	N	N	72	72	N
M. cerevisiae	MC-2	N	72	72	96	N	72	72	N
M. paucivorans	MPS-2	N	48	48	N	N	96	72	N
M. sueceinsis	MPS-2	N	48	72	N	ND[7]	ND	ND	ND

1- Incubation was carried out anaerobically at 30 ºC and time required to obtain a S/N ratio of > 2.0 was recorded
2- Control sample specified beer with no enrichment medium added
3- Beer+ MRS medium were taken in 1:1 ratio (v/v)
4- Beer + NBBC medium were taken in 1:1 ratio (v/v)
5- (85% SMMP media component: 15 % beer)
6- N- negative (S/N ratio < 2.0)
7- ND- Not detected

For beer only (control) samples, *P. cerevisiiphilus* was detected in 0, 2 and 4 % ABV beer samples after 72 hours of incubation with a S/N ratio of 4, 2.8 and 2.5 respectively using the PCFH-2 probe. *P. cerevisiiphilus* was not detected in 6.6 % ABV beer even after 96 hours of incubation. For MRS and NBB enriched beer 0, 2 and 4% beer showed positive results for detection after 48 hours of incubation, while SMMP enriched 0, 2 and 4 % ABV beer required 72 hours or more before a positive was obtained. Target bacteria were not detected in SMMP-enriched 6.6 % ABV beer even after 96 hours of incubation. *P. cerevisiiphilus* was however detected in MRS and NBB enriched 6.6 % beer after 96 and 72 hours respectively, with S/N ratio of 3.67 and 2.50 (using PCFH-2 probe).

Detection of *P. frisingensis* occurred sooner than *P. cerevisiiphilus* and *P. haikarae* in 0-4 % beers using the internal AE-labelled PCFH-2 probe. *P. frisingensis* was detected in 0, 2 and 4 % ABV beers after 48, 72 and 72 hours of incubation with a S/N ratio of 2.4, 2.7 and 2.65 respectively (using the PCFH-2 probe). *P. frisingensis* was not detected in 6.6 % beer but was detected in MRS and NBB-enriched 6.6 % ABV beer after only 48 hours of incubation. Detection of *P. frisingnesis* required more than 72 hours in SMMP enriched beers and it was not detected in SMMP enriched 6.6 % ABV beer.

P. haikarae showed the slowest growth among the beer spoilage *Pectinatus* species. Since the time required to detect *P. haikarae* in 0, 2 and 4 % beers was 96, 96 and 120 hours respectively. Thus a longer incubation time for *P. haikarae* in 4 % ABV beer was required compared to *P. cerevisiiphilus* and *P. frisingensis* in order to obtain a positive signal. The S/N ratio after 120 hours of incubation in 4 % ABV beer was 14, 26 and 2.9 for *P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae* respectively.

None of the beer spoilage *Megasphaera* species were detected in beer with 4 % ABV and above (using MC-2 and MPS-2 probes). In contrast, *M. cerevisiae*, was detected in MRS, NBB and SMMP-enriched 4% ABV beer after 72, 72 and 96 hours of incubation respectively (using MC-2 probe). *M. cerevisiae* was also detected in MRS and NBB - enriched 6.6 % ABV beer after 72 hours of incubation (using the MC-2 probe). In MRS and NBB-enriched 6.6 % ABV, *M. paucivorans* was detected after 96 and 72 hours of incubation respectively (using the MPS-2 probe). The results from beer enrichment experiments clearly showed that for beer less than 4% ABV, enrichment with MRS and NBB allowed detection of all the *Pectinatus* and *Megasphaera* species in after 48 -72 hours of incubation.

Table 4.15 Application of different detection methods to the brewing isolates and comprehensive comparison with HPA assay

Bacteria	SMMP [a]	Multiplex PCR [b]		Hybridisation Protection Assay [c]					RNA slot blot [d]	Partial 16S rRNA Gene sequencing [f]
		Pectinatus	*Megasphaera*	PC-2	PF-2	PH-2	MC-2	MPS-2		
P. cerevisiiphilus	+	+	-	+	-	-	-	-	+[1, 2]	ND [e]
P. frisingensis	+	+	-	-	+		-		+[2]	ND [e]
P. haikarae	+	+	-			+	-	-	+[3]	ND [e]
M. cerevisiae	+	-	+	-	-	-	+		+[4]	ND [e]
M. paucivorans	+	-	+	-	-	-	-	+	+[5]	ND [e]
M .sueceinsis	+	-	+	-	-	-	-	+	ND	ND [e]
Brewery isolates										
ICBD-PC-1	+	+	-	+	-	-	-	-	+[1, 2]	*P. cerevisiiphilus*
ICBD-PC-2	+	+	-	+	-	-	-	-	+[1, 2]	*P. cerevisiiphilus*
ICBD-PF-1	+	+	-	-	+	-	-	-	+[2]	*P. frisingensis*
ICBD-PF-2	+	+	-	-	+	-	-	-	+[2]	*P. frisingensis*
ICBD-PF-3	+	+	-	-	+	-	-	-	+[2]	*P. frisingensis*
ICBD-MC-1	+	-	+	-	-	-	+	-	+[3]	*M. cerevisiae*
ICBD-MC-2	+	-	+	-	-	-	+	-	+[3]	*M. cerevisiae*
ICBD-MC-3	+	-	+	-	-	-	-	-	+[3]	*M. cerevisiae*
brewery isolate-1	+	-	-	-	-	-	-	-	-	*Shigella flexineri*
brewery isolate-2	+	-	-	-	-	-	-	-	-	*Escherichia coli*
Specificity (%)	80	100	100	100	100	ND	100	ND	75	

a- SMMP broth was used (Dull *et al.*,1998) followed by single colony isolation on SMMP agar
b- Multiplex PCR was carried out as described by in section 2.5 (see materials and methods)
c- internal AE- labelled species specific probes were used except a single probe (MPS-1) was used for detection of *M. paucivorans* and *M. sueceinsis*
d- 5' DIG labelled probes of same sequence as probes in the HPA assay were used; 1- PC-2, 2- PF-2, 3- PH-2, 4- MC-2 and 5- MPS-2 probes
e- Not determined
f- confirmation based on partial rRNA gene sequence and nucleotide BLAST search (Johnson *et al.*, 2008)

3.22 Detection of brewery isolates of *Pectinatus* and *Megasphaera*

Putative isolates of *Pectinatus* or *Megasphaera* from brewery environments were obtained in 1 ml aliquots, frozen in 2 ml cryotubes (Fisher- Thermo Scientific) using liquid nitrogen and stored at -70 º C. Anhydrous DMSO (5-10 %) was used as a cryoprotectant. Frozen *Pectinatus* and *Megasphaera* (50-100 µl) were revived in 5 ml of SMMP broth incubated anaerobically at 30 ºC for 4 days, and then 100 µL of revived sample in SMMP broth was inoculated onto SMMP agar medium and incubated at 30 ºC for 4-7 days. Between 5 and 10 representative colonies of each of the *Pectinatus* and *Megasphaera* isolates were picked from each individual plate and further sub- cultured and maintained in PYF broth.

The samples were analysed using the multiplex PCR method (section 3.5), slot blot (section 3.18) and HPA assay (section 3.16) using the designed probes. The isolates were also confirmed by partial 16 S rRNA gene sequencing. Out of 10 bacterial isolates obtained from the SMMP plates, 2 and 3 bacterial isolates were confirmed as *P. cerevisiiphilus* and *P. frisingensis* respectively through partial 16S RNA gene sequencing, while three samples were confirmed as *M. cerevisiae*. Two isolates were identified as *Shigella flexineri* (99 %) and *Escherichia coli* each (99%). The details of comprehensive comparisons of bacterial detection methods and HPA are given in the Table 4.15.

Multiplex PCR and designed HPA assay were able to distinguish all 10 brewery isolates, detecting *Pectinatus* and *Megasphaera* specifically at species level giving 100 % specificity. RNA slot blot analysis showed 75 % specificity and was unable to distinguish between *P. cerevisiiphilus* and *P. frisingensis*. Overall SMMP medium was found to be 80 % specific for isolation of *Pectinatus* and *Megasphaera* species.

CHAPTER 5: DISCUSSION

Chapter 5 Discussion

5.1. Part I- Occurrence of anaerobic beer spoilage bacteria in major UK breweries

During the investigation of anaerobic beer spoilage bacteria in 10 major UK breweries, 117 samples were analysed. Of these 117 samples, two samples were positive for *P. cerevisiiphilus*; four samples were positive for the presence of *P. frisingensis*, two samples showed the presence of *M. cerevisiae* and one sample was found positive for the presence of *M. sueciensis* and *M. paucivorans* (detected by same pair of primers). *L. brevis* and *L. lindneri* were found to be the most frequently occurring *Lactobacillus* species with 16 and 13 positive samples respectively, while *L. casei*, *L. plantarum* and *L. corynformis* were found in 3, 1 and 1 samples respectively. Ten actual beer samples were positive for the presence of *Lactobacillus* species mainly from conditioning areas and filtration units. *Pediococcus* multiplex resulted in 24 positive samples for *Ped. damnosus/ Ped inopinatus*. Out of 24 samples positive for *Ped. damnosus/ Ped. inopinatus*, three samples were found from direct beer samples and the remaining samples were found positive for indirect samples. Additionally two yeast samples were received from brewery 3; both the samples were found to be positive for *Ped. damnosus* and one sample was positive for the presence of *L. paracollinoides*. The identity of these samples was later confirmed using partial 16S ribosomal gene sequencing.

It has been observed that beer with low alcohol content is more prone to spoilage by *Pectinatus* and *Megasphaera* species. *Pectinatus* species are more resistant to acidic pH and can survive at a pH of 4.1(Haikara and Lounatmaa, 1987). The pH tolerance of these anaerobic bacteria is influenced by the presence of ethanol (Suzuki 2011). *Pectinatus* and *Megasphaera* species are tolerant to hop bitter substances and can grow in beer with bitterness ranging between 33-38 EBC bitterness (Back, 1981; Kirchner *et al.*, 1980). *P. frisingensis* shows significant ability to maintain internal homeostasis to mild heat treatment (Tholazan *et al.*, 1999) and also its thermal resistance is high compared to *P. cerevisiiphilus* (Flahaut *et al.*, 2000). The growth of *Pectinatus* species is significantly affected by the oxygen content of beer and has been observed at a dissolved oxygen content of 1.91 mg/L (Soberka *et al.*, 1988). Modern filling techniques have limited the oxygen content of beer to 0.4-0.8 mg/L, which makes the growth and proliferation of *Pectinatus* in beer possible (Chowdhury *et al.*, 1995). The

growth of *Megasphaera* in beer with 3.5 % ethanol (w/v) is completely restricted (Haikara and Helander, 2006).

Routine pasteurisation of beer (27-30 PU) is sufficient to inhibit all microorganisms in the beer (Back, 1992). *Pectinatus* can be inhibited by heat treatment of 58-60 ºC for 1 min which is less than routine pasteurisation treatment (Haikara and Helander, 2006). Aseptic filtration of beer with 0.45 µm filters is as effective as flash pasteurisation (Back 1992). It has been reported that *Pectinatus* and *Megasphaera* are susceptible to most disinfectants used in the breweries such as iodine, chlorine and formaldehyde (Haikara and Helander, 2006). *Pectinatus* and *Megasphaera* are easy to control via thermal and disinfectant treatment but these microorganisms still survive in hard to access corners or biofilms which are not easy to access and disinfect (Suzuki, 2011).

Cleaning and hygiene validation of fermentation tanks, beer storage tanks and packaging lines is carried out by using an ATP bioluminescence method in 8 out of the 10 breweries. The sensitivity of the ATP method is not suitable for detection of low levels of contaminants; moreover some residues of cleaning agents and disinfectants could affect the enzyme reaction causing light production thus giving non-specific results (Lappalainen *et al.,* 2000). ATP bioluminescence is not suitable for actual detection of contaminants in breweries as the results are often not similar to those obtained by conventional methods for the same samples (Odebrecht *et al.*, 2000).

In recent years compared to premium lager, there has been development of sub-premium lager (4 % ABV) brands with low alcohol content and also mid strength lager. These brands are at increased risk from secondary contaminants including *Pectinatus*, primarily due to the low alcohol content of the beer. If these brands are brewed and packaged in the same conditions observed during the current study the potential risk of contamination in the final packaged product cannot be denied.

The presence of *Pectinatus* species on conveyor belts and star wheels of beer filling lines signifies a higher risk for packaged beer (Haikara and Helander, 2006). *Pectinatus* can be transmitted to fillers and subsequently to packaged beer via aerosols produced during the filling process (Durr, 1983) and cleaning procedures (Holah, 1992; Suzuki, 2011). CO_2 recovery systems are never subjected to cleaning regimes as they involve intensive dismantling of equipment (Lawrence, P. personal communication), hence the bacteria can prevail in this part of the brewery throughout the year creating a potential threat to packaged beer products in several ways.

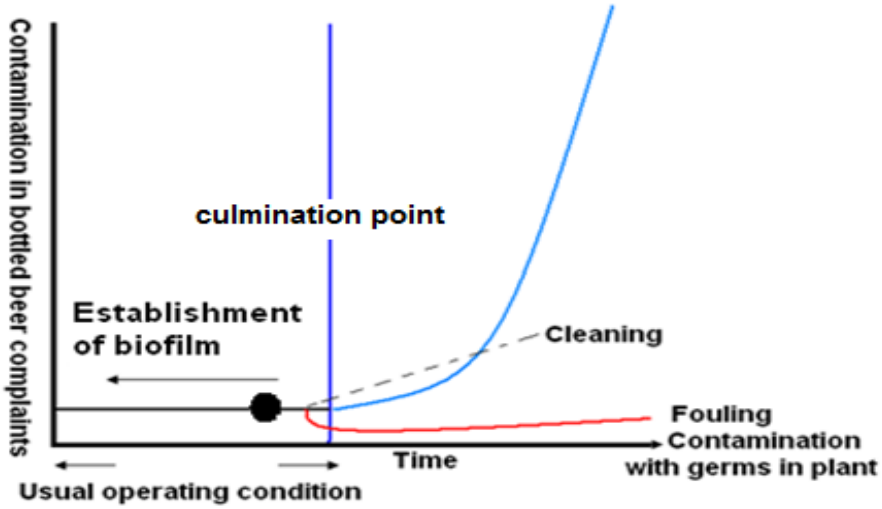


Figure 5.1 Relation between arousal of complaints and microbial nature of beer packaging lines (modified figure originally described by Back, 1994).

The presence of *Pectinatus* and *Megasphaera* in highly aerobic brewery environments can be due to formation of biofilms and symbiotic adaptations of microorganisms that survive within them (Suzuki, 2011). Their presence in highly aerobic conditions provides basic knowledge about the complexity of these biofilms. It is thought that anaerobic bacteria dwell in well established biofilms (Back, 1994; Timke *et al.*, 2005). Contamination could also occur from drainage areas or defective floors which are often niches of these anaerobic bacteria (Back, 2005; Motoyama 2003). Even though extensive cleaning procedures are adopted periodically in all the breweries, the cleaning procedures are not effective enough to completely remove attached biofilms and thus strictly anaerobic beer spoilage bacteria can propagate and be dispersed in packaging plants. The hygiene around filling machines is also important. The lack of any complaints of spoilage signifies that these secondary contaminants in bottling lines are still in their lag phase of adaptation due to periodic cleaning regimes ensuring hygienic operating conditions as described by Back (1994). However ineffective cleaning procedures (as we have found in sampled breweries), allow the continued presence of these microbes in the filling hall resulting in their concentration approaching culmination point (Figure 5.1). Subsequently some breweries can suffer severely from secondary contaminants without any noticeable prior warning (Back 1994).

Other possible reasons for no reports on anaerobic bacterial contamination in these breweries could be that most of the premium lagers (5 % ABV) brewed does not support growth of *Pectinatus* and *Megasphaera* and the presence of these anaerobic beer spoilers in aerobic brewery environments suggests that water could be the major source of contamination, but still the risk contamination of unpasteurised or flash pasteurised with low alcohol beer cannot be denied.

At present automated CIP (Cleaning in Place) with varying concentration of sodium hydroxide (NaOH; 1-2 %), cold and hot CIP, once or twice a week is utilised in most of the breweries (Table 4.2). Benefits of acid CIP over caustic CIP have been previously illustrated (Colosia, M., 2004). In some breweries the practice of increasing caustic concentration (1.5-4 %) along with increased temperature in hot CIP is also employed in case of severe problems of secondary contaminants, but this practice seems to be insignificant as there is need of modification in detergent formulation rather than using high concentration of caustic CIP which could be cost intensive.

It may be concluded that alternation in caustic CIP with the use of modified detergent formulations can be beneficial to achieve satisfactory hygiene conditions in breweries and packaging facilities. There is scope for development of modified detergent formulation as the trend in shifting from caustic CIP to alternate formulations can be seen in major lager breweries in the UK. Utilisation of SMMP medium (Dull *et al*., 1998) for detection of *Pectinatus* and *Megasphaera*) and ABD medium (Suzuki, 2008a) for detection of hard to culture lactic acid bacteria could be effective for detection of these microorganisms as an alternative the existing detection methods.

5.2. Discussion Part II Characterisation of putative isolates of *Pectinatus* and *Megasphaera*

Two putative isolates of *P. cerevisiiphilus*, three isolates of *P. frisingensis* and three isolates of *M. cerevisiae* isolated from the sampled breweries were characterised. All the isolates were Gram stained, checked for catalase and oxidase activity. The sugar utilisation profile and antibiotic susceptibility of these putative isolates for *Pectinatus* and *Megasphaera* were also determined. The presumed isolates were also characterised morphologically obtaining Scanning electron micrograph images and also by determining growth parameters on routine media. Genetic characterisation was carried out by partial sequencing of 16 S ribosomal gene. The morphological and physiological characterisation of the presumed *Pectinatus* and *Megasphaera* isolates showed good agreement on their identity.

For phylogenetic analysis of putatative isolates of *Pectinatus*, partial 16S ribosomal DNA gene sequences of validated type strains of *P. cerevisiiphilus* (ATCC 29359^T), *P. frisingensis* (ATCC 33332^T), *P. haikarae* (VTT-E 88329^T) and *P. portalensis* (strain B-6^T) were obtained from NCBI database. The phylogenetic trees using maximum parsimony (MP) were prepared using SEAVIEW version 4 software (Gouy *et al*., 2010). The phylogenetic analysis of *P. cerevisiiphilus* and *P. frisingensis* isolates has been illustrated in Figure 5.2 and 5.3 respectively. The maximum parsimony phylogenetic analysis of *M. cerevisiae* isolates is shown in Figure 5.4.

Presumed *P. cerevisiiphilus* isolates ICBD strain PC-1 and PC-2 were rooted in the same branch (65 % bootstrap replications for MP analysis) which was further rooted with *P. cerevisiiphilus* (ATCC 29359) (MP 100%). *P. haikarae* was further rooted with the above cluster (MP 96 %). *P. portalensis* and *P. frisingensis* were branched distantly (< 50 % MP).

Putative *P. frisingensis* isolates ICBD strain PF-1 and PF-2 formed a well supported group (MP 100 % bootstrap value), presumably because they are the same organism isolated from the same brewery (brewery 5). The grouped isolates (ICBD strain PF-1 and PF-2) were distantly placed from a cluster (MP 81 %) formed by *P. frisingensis* along with closely placed *P. cerevisiiphilus* and *P. haikarae* (MP 100 %).

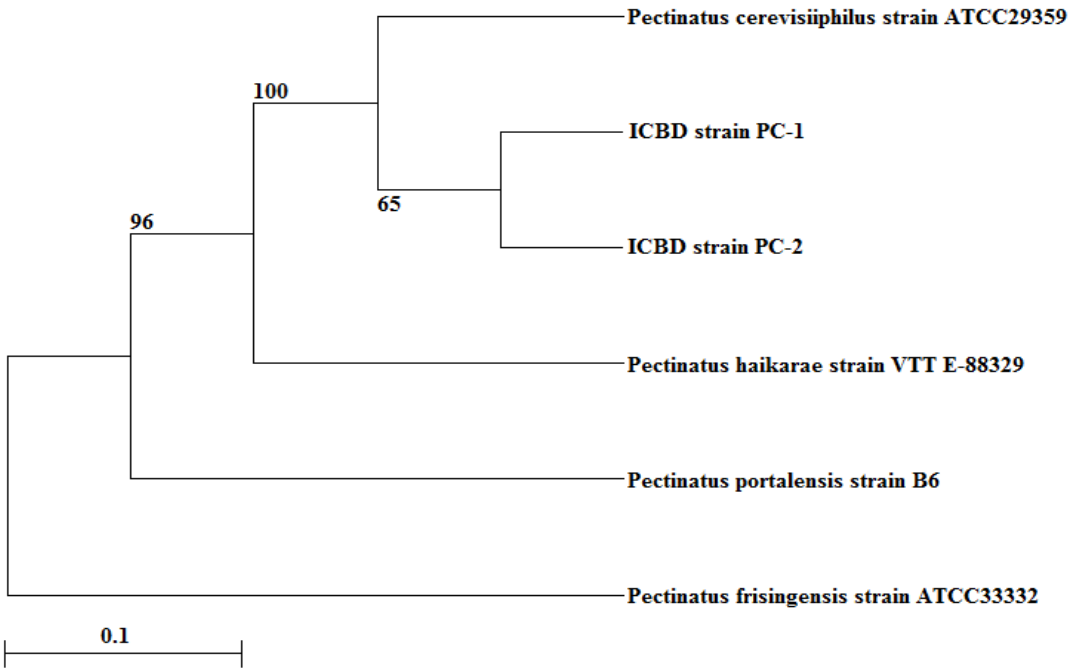


Figure: 5.2 Maximum-parsimony phylogenetic trees of 16S rRNA genes of *P. cerevisiiphilus* isolates from this study and validated beer spoilage *Pectinatus* species. Numbers along branches indicate bootstrap values (expressed as percentages of 100 replications). Scale bar 10 indicates nucleotide substitutions per site.

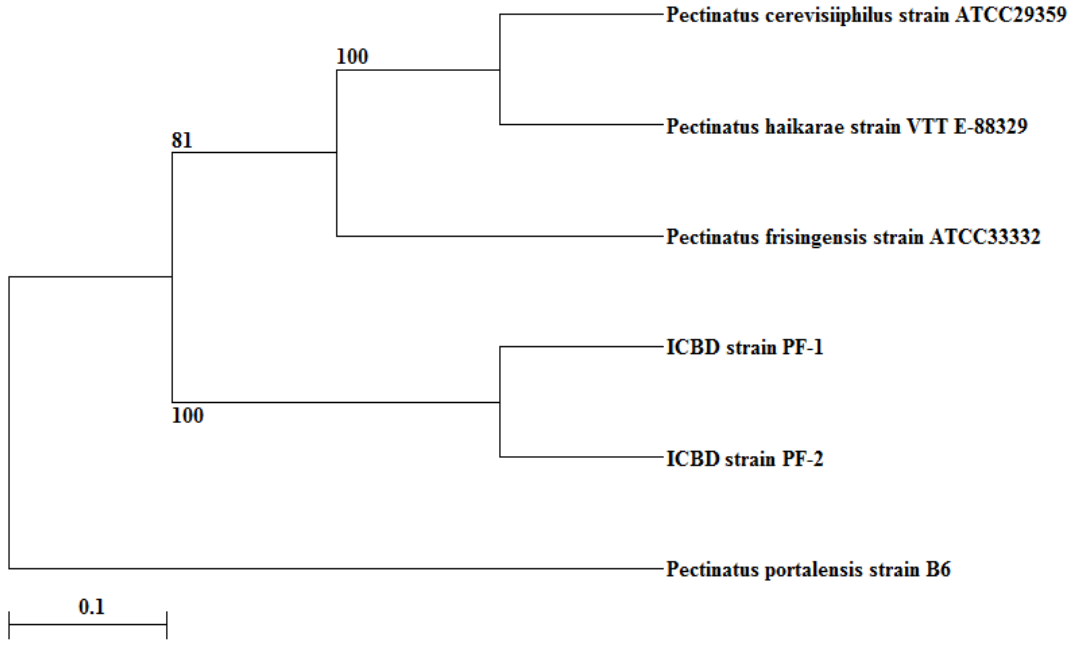


Figure: 5.3 Maximum-parsimony phylogenetic trees of 16S rRNA genes of *P. frisingnesis* isolates from this study and validated ***beer spoilage*** **Pectinatus** species. Numbers along branches indicate bootstrap values in percentages (100 replications). Scale bar indicates 10 nucleotide substitutions per site.

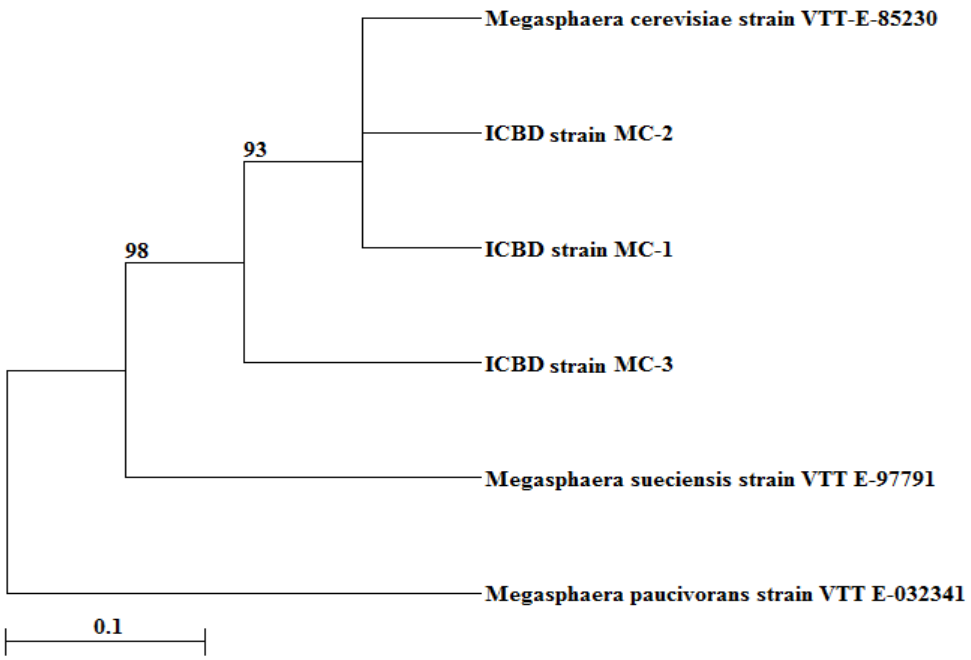


Figure: 5.4 Maximum-parsimony phylogenetic tree of 16S rRNA genes of *M. cerevisiae* isolates from this study and culture collection strains of beer spoilage *Megasphaera* species. The numbers at the nodes are percentages indicating the levels of bootstrap support (for 100 replications. Scale bar indicates 10 nucleotide substitutions per site.

The 16S ribosomal gene sequences of validated type strains of beer spoilage *Megasphaera* species *M. cerevisiae* (VTT-E 85230), *M. paucivorans* (VTT-E 97971) and *M. sueceinsis* (VTT-E 032341) were obtained from NCBI database. Presumed *M. cerevisiae* isolates ICBD strain MC-1 and MC-2 were grouped together with *M. cerevisiae* VTT-E 85230 (MP 93 %). The above group was further clustered to ICBD strain MC-3 with strong support (MP 98 %). Phylogenetically ICBD strains MC-1 and MC-2 differed from *M. sueceinsis* and *M. paucivorans* (MP <50 %).

All the *Pectinatus* and *Megasphaera* isolates showed strong ability to spoil low alcohol beer, and the presence of these microorganisms in the brewery environments could be a potential threat to low alcohol and unpasteurised beer. These isolates could prove threat to aseptically packaged beer which is flash pasteurised and sterile filtered before packaging. *Pectinatus* species were considered brewery related microorganisms, recently due to discovery of new *Pectinatus* species, their habitat has widened to other fermentation processes. Characterisation of putative strains and record of anaerobic microbes and their sampling sites could provide beneficial data for further studies and the experimental results could be useful in designing improvements in the UK breweries.

5.3. Discussion Part III- Hybridisation Protection Assay

5.3.1. Introduction

The Hybridisation Protection Assay (HPA) can be developed to detect nucleic acid, both DNA and RNA (Arnold *et al*., 1989; Arnold and Nelson, 1999; Hogan, 2000; Goto *et al*., 2002; Marlowe *et al*., 2003). Acridinium ester derivatives have also been utilised in immunoassays (Weeks *et al*., 1983; Natrajan *et al.,* 2011; Natrajan and Sharp, 2013; Roda and Guardgli, 2012; Zomer *et al.,* 2011; Brown *et al.,* 2009; Zhao *et al*., 2009). 16S ribosomal RNA (rRNA) is found in abundance in bacterial cells, often encoded by multi gene families and operons (Janda and Abbot, 2007). 16S rRNA is a large molecule (1500 bases) a part of which is highly conserved phylogenetically in bacterial cells (Oerther and de los Reyes, 2001). In non-viable bacterial cells ribosomal RNA starts degrading rapidly due to starvation (Zundel *et al*., 2009) and other physiological factors, and an assay based on detection of rRNA could therefore be a good indicator of cell viability.

There has been an argument about stability of rRNA and it has been previously demonstrated that rRNA could withstand different thermal and chemical treatments (Weber *et al.*, 2008). The precursors of rRNA are less stable than mature rRNA due to ease of access to the site of action RNAses in the less complex structure (Deutscher, 2006). However, although use of RNA precursor could be more effective in developing a sensitive detection assay, there is a lack of RNA handling facilities in brewing laboratories. It would therefore be more advantageous to develop an assay based on stable, abundantly available and robust target nucleic acid: rRNA has always been a choice for development of hybridisation-based detection methods (Mozola, 2000). For beer spoilage *Pectinatus* and *Megasphaera* species a large amount of data is available in public databases relating to 16S ribosomal genes (Yasuhara *et al*., 2001), compared to other potential target nucleic acid. This was the main reason for using ribosomal RNA as a target for developing an HPA using AE-labelled DNA probes. All the probes designed were based on phylogenetically conserved region of the 16S rRNA gene.

5.3.2. Probe selection and modification

AE-labelled probes could be designed as DNA, RNA or modified oligonucleotide according to the specific need of application of the HPA assay. DNA probes are more stable than RNA probes and are easy to handle. RNA probes are more susceptible to nuclease activity rendering them inactive (Mazumdar *et al.*, 1998), Use of modified oligonucleotides such as 2'-O-methly-oligoribonucleotide which are a naturally occurring modification of RNA have also been described in a HPA assay (Mazumdar *et al.*, 1998). These molecules are highly nuclease resistant and have high affinity towards RNA molecules (De Mesmackera *et al*., 1995; Lamond and Sproat, 1993) making them suitable for HPA assays (Mazumdar *et al*., 1998). The main reason for selecting single stranded DNA as a probe was due to the fact that the stability of the AE molecule is greatly increased in a mixed duplex (RNA–DNA hybrids) (Becker *et al*.,1999), and as rRNA was already selected as a target it was logically advantageous to use DNA as a probe.

Various strategies have been described regarding modification of nucleic acid probes (Arnold *et al*., 1989; Arnold *et al*., 2000). The most common is introducing an amine group at the end 3' or 5' of the probe using an amine linker arm (Arnold *et al*., 2000). Nucleotide base replacement with an amine-modified nucleotide during synthesis can be another alternative (Arnold *et al*., 1989). After synthesis, chemical modification of nucleotide bases to introduce a reactive amine group has also been described (Viscidi *et al*., 1986). Alkyl amine derivatives of nucleotide base linking phosphates can be used to modify nucleic acid probes, and the amine group of such probes can be later labelled with AE at multiple sites. For the present study two types of AE labelled probes were developed: 5' amine modified AE probes and internal AE labelled probes using an amine modified thymidine base.

5.3.3. Comparison of Developed HPA assay and RNA slot blot analysis

The designed internally AE-labelled probes were able to detect target RNA within the range of 0.016-0.0032 pmol where as sensitivity of RNA slot analysis using DIG labelled probes was limited to 0.4-.08 pmol. All AE labelled probes showed high specificity. The specificity and sensitivity of all the DIG labelled probes was found to be satisfactory except for the PC-DIG probe which gave lower intensity signals for high amounts of RNA (2.0 and 0.4 pmol) isolated from *P. frisingensis*

Comparison of conventional membrane hybridization (Southern blots and Northern blots) using radio labelled DNA probes with HPA assay has been described previously (Arnold *et al*., 1989; Dhingra et al., 1999; Nelson and McDough, 1990). RNA slot blot analysis was carried out for all the designed DNA probes for *Pectinatus* and *Megasphaera* species, using probes labelled with DIG at the 5' end. All the DIG labelled probes were able to detect down to 0.4 to 0.08 pmol of target RNA, The DIG labelled PCFH-DIG, PH-DIG and MPS-DIG probes gave less intense signal for 0.08 pmol of target RNA compared to DIG labelled PC-DIG, PF-DIG and MC-DIG which gave clear detection signals at this concentration. None of the DIG labelled probes could detect target RNA at 0.016 pmol or below.

For the given study 5' DIG labelled probes were used. The sensitivity of RNA slot blot could have been increased by using various other methods for DIG labelling, such as incorporation of digoxigenin-dUTP (uridine tri phosphate) through *in vitro* amplification methods. As the length of the probes used in this study were 26-27 bp, the use of *in vitro* amplification method would not have been possible, hence as an alternative end labelled probes were used.

5.3.4. Bacterial CFU detection limit of HPA assay

All the designed probes showed high specificity towards target RNA and could detect bacterial contamination within the range of ca. $5x10^2 – 1x10^3$ CFU using HPA assay. Internal AE-labelled probes PC-2, PF-2 and MC-2 were able to detect $5x\ 10^2$ CFU of target bacteria but sensitivity of PH-1 and MPS-2 probes was limited to $1x10^3$ CFU. Genus specific internal AE-labelled PCFH-2 probe was able to detect $5x\ 10^2$ CFU of RNA stabilised frozen pellet of *P. cerevisiiphilus, P.frisingensis and P. haikarae*

Detection limits within the range of 10^4-10^5 CFU have been documented (Clancy *et al*., 2012; Ninet *et al*, 1992) and detection of bacterial contamination ranging from 10^2-10^3 CFU/ml using a commercial HPA kit in clinical samples has also been recorded (Brecher *et al*., 1994). Most of the described HPA in the literature are clinical applications of commercial HPA based kits; the cut off values for these kits is placed higher than statistical values in order to avoid the risk of false positive results which may be a great concern in clinical laboratories (Lindholm and Sarkkinen, 2004). Compared to clinical and food-derived microbiological samples, a very narrow range of spoilage microorganisms are able to spoil beer due to various antimicrobial factors in

beer such as presence of ethanol, low pH, presence of hop bitterness compounds and low level of nutrients (Suzuki, 2011; Vriesekoop *et al*., 2013). *Pectinatus* and *Megasphaera* species comprised a small group of beer spoiling organisms compared to complex and phylogenetically well studied clinical pathogens, hence the cut-off values for the present study remained lower compared than other studies giving higher detection sensitivity.

5.3.5. Performance of HPA with beer enrichment media using different ABV beers

The results from beer enrichment experiments clearly showed that for beer less than 4% ABV, enrichment with MRS and NBB allowed detection of all the *Pectinatus* and *Megasphaera* species in after 48 -72 hours of incubation.

Previously an effect of media components on the performance of HPA assay has been documented (Stahl and Amann, 1991; Ninet *et al.,* 1993 and Partis *et al.,* 1994). In the present all three enrichment media were shown to be compatible with HPA assay and no inhibitory effects of media components on the HPA were observed.

All the beer spoilage *Pectinatus* and *Megasphaera* were detected in 6.6 % ABV beer enriched with MRS and NBB but failed grow in SMMP-enriched beer. *Pectinatus* species can grow in beer with ethanol concentration of 3.7 to 4.4 % (w/v) whereas the growth of *Megasphaera* species is completely inhibited at ethanol concentration of 3.5 % (w/v). The effective alcohol concentration of beer enriched with MRS and NBB media (1:1 ratio (v/v)) becomes half, where as for SMMP medium the effective alcohol content of the beer was reduced by only 15 % less, hence beer spoilage *Pectinatus* and *Megasphaera* species were able to grow in the beer 6.6 % ABV enriched with MRS and NBB, but not SMMP medium.

5.3.6. Detection of brewery isolates of *Pectinatus* and *Megasphaera*

The HPA assay developed was applied for detection of putative isolates of *Pectinatus* and *Megasphaera* from brewery environments and the same samples were analysed using SMMP medium (Lee, 1994; Dull *et al.*, 1998), multiplex PCR method (Asano *et al*., 2008, Suzuki *et al*, 2008), RNA slot blot analysis and partial 16S rRNA gene sequencing for comprehensive comparison of detection methods used.

Multiplex PCR and designed HPA assay were able to distinguish all 10 brewery isolates, detecting *Pectinatus* and *Megasphaera* specifically at species level giving 100 % specificity. RNA slot blot analysis showed 75 % specificity and was unable to distinguish between *P. cerevisiiphilus* and *P. frisingensis*.

Overall SMMP medium was found to be 80 % specific for isolation of *Pectinatus* and *Megasphaera* species. Although SMMP could be selective in growth of *Pectinatus* and *Megasphaera* species, presence of ethanol in SMMP medium is an inhibitory factor for growth of *enterobacteraeceace* (Lee, 1994), survival of *E.coli* in mid strength beer has been previously documented (Menz *et al*., 2010). In the case of SMMP agar, autoclaving leads to loss of ethanol which needs to be adjusted, and growth of enterobacteria is possible if the ethanol concentration is not adjusted properly.

Multiplex PCR (Asano *et al*., 2008; Suzuki *et al*., 2008) and real time PCR (Juvonen *et al*., 2009) methods with enhanced detection limits (1-10 CFU/ 100ml) have been developed but their application in brewing laboratories is limited due to their high cost to benefit ratio. Advanced detection media have also been developed recently to detect *Pectinatus* and *Megasphaera* species in real time (Matoulková *et al*., 2012) but a single medium could not detect all the beer spoilage bacteria. The developed HPA method could be effectively applied in brewing laboratories as it is a simple and economical substitute for present methods. In last decade, highly stable acridinium ester derivatives have been synthesised (Brown *et al*., 2009; Razavi and McCapra, 2000) which has made handling and performance of HPA assay even easier. Moreover, the data obtained from HPA assay are quantitative and hence easy to analyse compared to qualitative data which could be wrongly interpreted. A luminometer is the only instrument required for HPA assay and RNA handling has been made easy due to stabilisation reagents available commercially. The method described in this study could also be implemented with enrichment media to effectively reduce detection time in the case of a low level of contaminants.

5.4. Conclusion and further study

From the preliminary study conducted to investigate occurrence of *Pectinatus* and *Megasphaera* along with *Lactobacillus* and *Pediococcus*, it can be concluded that *Lactobacillus* and *Pediococcus* constitute the major problems in the sampled breweries. Despite being obligatory anaerobes, *Pectinatus* and *Megasphaera* were found in aerobic brewery environments, and though these bacteria are not a major concern, their occurrence in aerobic brewery environments indicates sanitation problems and revealed the presence of highly established biofilms in some breweries. The *Pectinatus* and *Megasphaera* isolates showed beer spoilage ability. These isolates could prove threat to aseptically packaged beer which is flash pasteurised and sterile filtered before packaging. *Pectinatus* species were considered brewery related microorganisms, recently due to discovery of new *Pectinatus* species, their habitat has widened to other fermentation processes. Utilisation of SMMP medium (for detection of *Pectinatus* and *Megasphaera*) and ABD medium (for detection of hard to culture lactic acid bacteria) could be effective for detection of these microorganisms as an alternative to rapid detection methods.

In recent years due to growth in production of beer with reduced antimicrobial hurdles, brewery hygiene has become more important. The reliable rapid detection of beer spoilage microorganisms can expedite troubleshooting in the breweries. The HPA assay developed in this study showed high specificity towards target microorganisms and could detect bacterial contaminants down to $5x10^2$- $1x10^3$ CFU. Although developed HPA has shown high specificity, sample enrichment with bacterial growth media and incubation for 2-3 days can enhance the reliability of the assay. Enrichment with MRS and NBB media enhanced the detection speed of the HPA assay. All internal- AE labelled probes showed high sensitivity towards target bacteria and were able to distinguish the target bacteria with specificity.

Although a number of rapid detection methods are available, their application in the brewing industry has been limited primarily due to high cost and complexity of the techniques. ATP bioluminescence hygiene monitoring technique is widely used due to its simplicity and quantitative data output. HPA assay formats are simple and rapid, and the entire assay can be performed within 1-2 hours hence a large number of samples can be processed within a short period of time.

The data available for HPA assay are quantitative, reliable and reproducible hence the results are easy to analyse. The HPA assay can be applied to the detection of DNA and RNA molecules from diverse sources; hence these assay formats are versatile and show compatibility with different forms of samples. This study demonstrates the potential of the HPA assay in brewing laboratories as a sensitive method for detection of beer spoilage microorganisms and can be further extended to detect other beer spoilage microorganisms, especially beer spoilage *Lactobacillus* and *Pediococcus*. Further research is needed to adapt a hand-held luminometer to conduct the HPA assay.

APPENDICES

APPENDIX- I List sampling points and multiplex PCR results

S. No.	Sample site description	sample type	Multiplex PCR results			
			Pectinatus	***Megasphaera***	***Lactobacillus***	***Pediococcus***
brewery no. 1						
1	Bottling line conveyor belt (in-feed)	sterile swab			*L.brevis*	
2	Bottling line conveyor belt (out- let)	sterile swab				*Ped. damnosus*
3	Bottling line star-wheel (in-feed)	sterile swab				
4	Bottling line star-wheel (out- let)	sterile swab	*P. cerevisiiphilus*		*L.brevis*	
5	Bottling line filler rinse	liquid rinse			*L.brevis*	
6	Bottling line crowner	sterile swab				
7	Bottling line jetter	sterile swab			*L.brevis*	*Ped. damnosus*
8	Connecting hose pipe (internal)	sterile swab				*Ped. damnosus*
9	Bright beer tank sample point	beer sample			*L. brevis*	
10	Unpasteurised bottled beer	beer sample				
brewery no. 2						
11	Canning line conveyor belt	sterile swab				
12	Canning line conveyor belt	sterile swab				*Ped. damnosus*
13	Canning line conveyor belt	sterile swab	*P. frisingensis*	*M. cerevisiae*		
14	Canning line conveyor belt	sterile swab	*P. frisingensis*		*L. brevis*	*Ped. damnosus*
15	Bright beer holding tank (canning)	beer sample			*L. brevis*	
16	Keg filling filler head	sterile swab			*L. brevis*	
17	Bright beer tank	beer sample				
18	Bright beer buffer tank (keg line)	beer sample				

19	Bright beer buffer tank (keg line	beer sample				
20	Seamer of canning line	sterile swab				
brewery no. 3						
21	bottling line conveyor belt	sterile swab				
22	bottling line star-wheel	sterile swab			*L. brevis*	*Ped. damnosus*
23	bottling line star-wheel	sterile swab				*Ped. damnosus*
24	bottling line star-wheel	sterile swab	*P. cerevisiiphilus*	*M.c, Mp/Ms**		*Ped. damnosus*
25	bottling line jetter	sterile swab			*L. lindneri*	
26	Bottling line crowner	sterile swab			*L. brevis*	
27	bottling line conveyor belt	sterile swab				
28	bottling line filler	beer sample				
29	Pre-pasteurised beer from the fillers	beer sample			*L. lindneri*	
30	CO_2 out let from fermentors	liquid sample				
brewery no. 4						
31	Bottling line inlet star wheel	sterile swab				
32	bottling line filler rinse	rinse sample				
33	bottling line conveyor belt	sterile swab			*L. brevis*	*Ped. damnosus*
34	bottling line star wheel	sterile swab				*Ped. damnosus*
35	bottling line star wheel	sterile swab				*Ped. damnosus*
36	Bottling line crowner	sterile swab			*L. lindneri*	
37	bottling line conveyor belt	sterile swab			*L. lindneri*	*Ped. damnosus*
38	bottling line jetter	sterile swab			*L. lindneri*	
39	fermentation vessel sampling point	beer sample				
40	Bright beer tank sample point	beer sample				

brewery no. 5					
41	bottling line conveyor belt	sterile swab			*Ped. damnosus*
42	bottling line conveyor belt	sterile swab			
43	bottling line star wheel	sterile swab			
44	Bottling line filler rinse	beer sample			*Ped. damnosus*
45	bottling line conveyor belt	sterile swab			*Ped. damnosus*
46	bottling line conveyor belt	sterile swab		*L.brevis*	*Ped. damnosus*
47	bottling line jetter rinse	sterile swab			
48	bottling line filler rinse	beer sample			
49	FFV- 33 CO2 collecting pods	rinse sample	*P. frisingensis*	*L. brevis*	
50	FFV- 08 CO2 collecting pods	rinse sample			
51	FFV- 59 CO2 collecting pods	rinse sample	*P. frisingensis*		
52	Bright beer tank sample point	beer sample			
53	Bright beer tank sample point	beer sample			
54	Ale yeast storage tank	yeast slurry			
55	Lager yeast storage tank	yeast slurry			
Brewery no. 6					
56	Fermentation vessel sampling point	beer sample			
57	Fermentation vessel sampling point	beer sample			
58	Canning line seamer star wheel	sterile swab			
59	Canning line seamer star wheel	sterile swab			
60	Canning line conveyor belt	sterile swab			
61	Canning line conveyor belt	sterile swab			
62	Canning line filler rinse	rinse sample			
63	Canning line filler rinse	rinse sample			

64	Canning line conveyor belt	sterile swab	*Ped. damnosus*
65	Canning line in-feed star wheel	sterile swab	*L. brevis*
66	Canning line star wheel	sterile swab	
67	Canning line seamer out roller	sterile swab	
68	Pre-pasteurised canned beer	beer sample	
69	Bright beer tank sample point (64)	beer sample	
70	bright beer tank sample point (12)	beer sample	
Brewery no. 7			
71	Fermentation vessel (34)	beer sample	
72	Fermentation vessel (35)	beer sample	*L. plantarum*
73	Fermentation vessel (66)	beer sample	
74	Conditioning tank (66)	beer sample	
75	Conditioning tank (60)	beer sample	*L. lindneri*
76	bright beer tank sample point (12)	beer sample	
77	bright beer tank sample point (12)	beer sample	
78	Yeast storage tank	yeast slurry	
Brewery no. 8			
79	fermentation vessel (40)	beer sample	
80	fermentation vessel (26)	beer sample	
81	Conditioning tank	beer sample	
82	Conditioning tank	beer sample	
83	bright beer tank	beer sample	*L. lindneri*
84	bright beer tank	beer sample	
85	Yeast storage tank	yeast slurry	

86	Yeast storage tank	yeast slurry		
87	Pre-filtration beer tank sample point	beer sample	*L. coryniformis*	*Ped. damnosus*
88	Filtration area- Orion	beer sample		
Brewery no. 9				
89	Maturation tank sample point (209)	beer sample	*L. lindneri*	
90	Maturation tank sample point (211)	beer sample	*L. lindneri*	
91	Maturation tank sample point (215)	beer sample	*L. lindneri*	
92	bright beer tank sample point (308)	beer sample		
93	bright beer tank sample point (311)	beer sample	*L. casei*	
94	bright beer tank sample point (310)	beer sample		*Ped. damnosus*
95	filter bright beer 1	beer sample		
96	rough bright beer 1	beer sample	*L. lindneri*	
97	Recovered bright beer	beer sample		
98	Fermentation vessel- 10	beer sample	*L. brevis*	
Brewery no. 10				
99	Bottling line conveyor belt	sterile swab		*Ped. damnosus*
100	Bottling line conveyor belt	sterile swab		*Ped. damnosus*
101	bottling line star wheel	sterile swab		*Ped. damnosus*
102	bottling line star wheel	sterile swab		
103	bottling line star wheel	sterile swab		
104	bottling line star wheel	sterile swab	*L. lindneri*	
105	bottling line star wheel	sterile swab	*L. lindneri*, *L. casei*	
106	Bottling line crowner	sterile swab		
107	Bottling line crowner	sterile swab		

108	bottling line star wheel	sterile swab		
109	bottling line jetter rinse	rinse sample		
110	bottling line filler rinse (1)	rinse sample		
111	bottling line filler rinse (2)	rinse sample		
112	bottling line filler rinse (3)	rinse sample		
113	bottling line conveyor belt	sterile swab		*Ped. damnosus*
114	bottling line conveyor belt	sterile swab	*L. casei*	
115	bottling line filler rinse (4)	sterile swab		
116	bottling line conveyor belt	sterile swab		
117	bottling line filler rinse (5)	rinse sample		*Ped. damnosus*

* sample was positive for *M. cerevisiae* (mc) and *M .paucivorans (mp/ M. sueceinsis(ms)*

Appendix- II Sugar utilisation profiles for putative isolates of *Pectinatus* species

S.N	Substrate	DSM 20467	DSM 6306	DSM16980	ICBD PC-1	ICBD PC-2	ICBD PF-1	ICBD PF-2	ICBD PF-3
0		-	-	-	-	-	-	-	-
1	glycerol	w	-	-	+	w	+	+	w
2	erythritol	-	w	w	+	w	+	+	+
3	D- arabinose	-	-	-	-	-	-	-	-
4	L-arabonose	-	+	+	+	+	+	+	+
5	D-ribose	-	-	-	-	-	-	-	-
6	D-xylose	+	-	-	+	+	-	-	-
7	L-xylose	-	-	-	-	-	-	-	-
8	D-adanitoll	-	-	-	-	-	-	-	-
9	methyl-β-D-xylopyranoside		-	-	-	-	-	-	-
10	D-galactose	+	+	+	w	w	+	+	+
11	D- glucose	+	+	+	+	+	+	w	w
12	D- fructose	+	+	+	+	+	+	+	+
13	D- mannose	+	+	+	+	+	+	+	+
14	L- sorbose	-	-	-	-	-			
15	L- rhamonose	-	-	-	+	+	+	+	+
16	dulcitol	-	+	+	-	-	+	+	+
17	inositol	-	-	-	+	+	+	+	+
18	mannitol	-	+	+	+	w	+	+	+
19	sorbitol	-	-	-	+	w	+	+	+
20	methyl-α-D-mannopyranoside	-	-	-	-	-			

S.N	Substrate	DSM20467	DSM6306	DSM16980	ICBD PC-1	ICBD PC-2	ICBD PF-1	ICBD PF-2	ICBD PF-3
21	methyl-α-D-glucopyranoside	-	-	-	-	-	+	+	+
22	N-acetylglucosamine	-	-	-	-	-	+	+	+
23	amygdaline	-	-	-	-	-	-	-	-
24	arbutin	-	-	-	-	-	+	+	+
25	esculin	+	+	-	+	+	+	w	w
26	salicin	-	-	-	-	-	+	+	+
27	D-cellobiose	-	-	-	-	-	+	+	+
28	D-maltose	-	-	-	-	-	+	+	+
29	D-lactose	-	-	+	w	w	-	-	-
30	D-mellibiose	w	-	-	+	w	-	-	-
31	D-sucrose	-	-	-	-	-	-	-	-
32	D-trehalose	-	-	-	-	-	-	-	-
33	D-Enulase	-	-	-	-	-	-	-	-
34	D-Melezitose	-	-	-	-	-	-	-	-
35	D-raffinose	-	-	-	-	-	-	-	-
36	starch	-	-	-	-	-	-	-	-
37	glycogen	-	-	-	-	-	-	-	-
38	xylitol	-	w	w	-	-	-	-	-
39	D-gentiobiose	-	-	-	-	-	+	+	+
40	D-turanose	-	-	-	-	-	+	+	+
41	D-lyxose	-	-	-	-	-	w	-	w
42	D- tagatose	-	-	-	-	-	+	+	+
43	D-fucose	-	-	-	-	-	-	-	-
44	L-fucose	-	-	-	-	-	-	-	-
45	D-arabitol	-	-	-	-	-	-	-	-
46	L-arabitol	-	-	-	w	w	w	-	w

S.N.	Substrate	DSM20467	DSM6306	DSM16980	ICBD PC-1	ICBD PC-2	ICBD PF-1	ICBD PF-2	ICBD PF-3
47	pottasium gluconate	-	-	-	+	+	w	-	w
48	2-keto gluconate	-	-	-	-	-	-	-	-
49	5-keto-gluconate	-	-	-	w	w	-	-	-

(-) - negative result, (+) positive result, w- weakly positive.

DSM 20467- *P. cerevisiiphilus*, **DSM 6306**- *P. frisingnesis*, **DSM 16980**- *P. haikarae*,

ICBD PC-1, ICBD- PC-2- putative strains of *P. cerevisiiphilus*, **ICBD PF-1, ICBD PF-2 and ICBD PF- and ICBD PF-3**- putative strains of *P. frisingensis.*

Appendix III

Partial ribosomal gene sequences of isolates of *Pectinatus* and *Megasphaera*

III A Partial 16S ribosomal gene sequences (ICBD strain PC-1) aligned

```
Query  14   AGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA  73
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  567  AGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA  508

Query  74   GCGCAACCCCTATCATTTGTTGCCAGCACGTCAAGGTGGGAACTCAAATGAGACTGCCGC  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  507  GCGCAACCCCTATCATTTGTTGCCAGCACGTCAAGGTGGGAACTCAAATGAGACTGCCGC  448

Query  134  GGACAACGCGGAGGAAGGCGGGGATGACGTCAAGTCATCATGCCCCTTACGTCCTGGGCT  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  447  GGACAACGCGGAGGAAGGCGGGGATGACGTCAAGTCATCATGCCCCTTACGTCCTGGGCT  388

Query  194  ACACACGTACTACAATGGGATACACAGAGGGAAGCGAAGGAGTGATCTGGAGCGGAACCC  253
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  387  ACACACGTACTACAATGGGATACACAGAGGGAAGCGAAGGAGTGATCTGGAGCGGAACCC  328

Query  254  AAAAAATATCCCCCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATCGC  313
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  327  AAAAAATATCCCCCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATCGC  268

Query  314  TAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC  373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  267  TAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC  208

Query  374  GTCACACCACGAAAGTCATTCACACCCGAAGCCGGCTAAGGGCCGCAAGGAACCGACCGT  433
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  207  GTCACACCACGAAAGTCATTCACACCCGAAGCCGGCTAAGGGCCGCAAGGAACCGACCGT  148

Query  434  CTAAGGTGGGGGCGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCG  493
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  147  CTAAGGTGGGGGCGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCG  88

Query  494  GCTGGATCACCTCCTTTCTAAGGATTTGACAAAAATCTGTCGAGTACATCCGGAATATGT  553
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87   GCTGGATCACCTCCTTTCTAAGGATTTGACAAAAATCTGTCGAGTACATCCGGAATATGT  28

Query  554  ATTGTTTGGTTTTGAGG  570
            |||||||||||| ||||
Sbjct  27   ATTGTTTGGTTT-GAGG  12
```

III B Partial 16S ribosomal gene sequences (ICBD strain PC-2) aligned

```
Query  11   AACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA  70
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  568  AACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA  509

Query  71   CGAGCGCAACCCCTATCATTTGTTGCCAGCACGTCAAGGTGGGAACTCAAATGAGACTGC  130
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  508  CGAGCGCAACCCCTATCATTTGTTGCCAGCACGTCAAGGTGGGAACTCAAATGAGACTGC  449

Query  131  CGCGGACAACGCGGAGGAAGGCGGGGATGACGTCAAGTCATCATGCCCCTTACGTCCTGG  190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  448  CGCGGACAACGCGGAGGAAGGCGGGGATGACGTCAAGTCATCATGCCCCTTACGTCCTGG  389

Query  191  GCTACACACGTACTACAATGGGATACACAGAGGGAAGCGAAGGAGTGATCTGGAGCGGAA  250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388  GCTACACACGTACTACAATGGGATACACAGAGGGAAGCGAAGGAGTGATCTGGAGCGGAA  329

Query  251  CCCAAAAAAATATCCCCCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAAT  310
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328  CCCAAAAAAATATCCCCCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAAT  269

Query  311  CGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCGGGCCTTGTACACACCG  370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268  CGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCGGGCCTTGTACACACCG  209

Query  371  CCCGTCACACCACGAAAGTCATTCACACCCGAAGCCGGCTAAGGGCCGCAAGGAACCGAC  430
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208  CCCGTCACACCACGAAAGTCATTCACACCCGAAGCCGGCTAAGGGCCGCAAGGAACCGAC  149

Query  431  CGTCTAAGGTGGGGGCGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGT  490
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  148  CGTCTAAGGTGGGGGCGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGT  89

Query  491  GCGGCTGGATCACCTCCTTTCTAAGGATTTGACAAAAATCTGTCGAGTACATCCGGAATA  550
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  88   GCGGCTGGATCACCTCCTTTCTAAGGATTTGACAAAAATCTGTCGAGTACATCCGGAATA  29

Query  551  TGTATTGTTTGGTTT  565
            |||||||||||||||
Sbjct  28   TGTATTGTTTGGTTT  14
```

III C Partial 16S ribosomal gene sequences (ICBD strain PF-1) aligned

```
Query  1    CCGATACGCCTTCTCTGAGCCTCAGCAACGTCAGTTCGGACCCCATCTCGGGGTTGAGCC  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  676  CCGATACGCCTTCTCTGAGCCTCAGCAACGTCAGTTCGGACCCCATCTCGGGGTTGAGCC  617

Query  61   CCGGGCTTTTCAGATCCGCTTAATGTTCCGCCTGCGCTCCCTTTACGCCCAATGATTCCG  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  616  CCGGGCTTTTCAGATCCGCTTAATGTTCCGCCTGCGCTCCCTTTACGCCCAATGATTCCG  557

Query  121  GACAACGCTTGGCGCCTACGTATTACCGCGGCTGCTGGCACGTAATTAGCCGTGGCTTTC  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  556  GACAACGCTTGGCGCCTACGTATTACCGCGGCTGCTGGCACGTAATTAGCCGTGGCTTTC  497

Query  181  TAACGGGGTACCGTCATTCAATATACTGATTGGCTATAGTGCCGTTCGTCCCCTGCAACA  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  TAACGGGGTACCGTCATTCAATATACTGATTGGCTATAGTGCCGTTCGTCCCCTGCAACA  437

Query  241  GAACTTTACGATCCGAAGACCTTCCTCGTTCACGCGGCGTTGCTCCGTCAGGCTTTCGCC  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  GAACTTTACGATCCGAAGACCTTCCTCGTTCACGCGGCGTTGCTCCGTCAGGCTTTCGCC  377

Query  301  CATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376  CATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA  317

Query  361  ATGTGGCCGTTCATCCTCTCAGACCGGCTACTGATCGTCGCCTTGGTGCGCCGTTACCGT  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  ATGTGGCCGTTCATCCTCTCAGACCGGCTACTGATCGTCGCCTTGGTGCGCCGTTACCGT  257

Query  421  CACCAACCAGCTAATCAGACGGGGCCCATCTCCAAGCGATAGCTAAAAGCCACCTTTGGT  480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256  CACCAACCAGCTAATCAGACGGGGCCCATCTCCAAGCGATAGCTAAAAGCCACCTTTGGT  197

Query  481  AATACTGCCATGCATCAGTATTACAACATTCGGTATTAGCACCCCTTTCGGAGTGTTGTC  540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196  AATACTGCCATGCATCAGTATTACAACATTCGGTATTAGCACCCCTTTCGGAGTGTTGTC  137

Query  541  CCCATCTTGGAGGCAGGTTGCCTACGCGTTACTCACCCGTTTGCCACTAAGCCCTTACCG  600
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  136  CCCATCTTGGAGGCAGGTTGCCTACGCGTTACTCACCCGTTTGCCACTAAGCCCTTACCG  77

Query  601  AAATAAGAGCCTCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCGTCCAAGCCA  660
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76   AAATAAGAGCCTCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCGTCCAAGCCA  17

Query  661  GAAAAAAAAAAATAAT  676
           ||||||||||||||||
Sbjct  16   GAAAAAAAAAAATAAT  1
```

III D Partial 16S ribosomal gene sequences (ICBD strain PF-2) aligned

```
Query  1    CAGTTCGGACCCCATCTCGGGGTTGAGCCCCGGGCTTTTCAGATCCGCTTAATGTTCCGC  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  611  CAGTTCGGACCCCATCTCGGGGTTGAGCCCCGGGCTTTTCAGATCCGCTTAATGTTCCGC  552

Query  61   CTGCGCTCCCTTTACGCCCAATGATTCCGGACAACGCTTGGCGCCTACGTATTACCGCGG  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  551  CTGCGCTCCCTTTACGCCCAATGATTCCGGACAACGCTTGGCGCCTACGTATTACCGCGG  492

Query  121  CTGCTGGCACGTAATTAGCCGTGGCTTTCTTACGCGGTACCGTCCTTCAATATACTTATT  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  491  CTGCTGGCACGTAATTAGCCGTGGCTTTCTTACGCGGTACCGTCCTTCAATATACTTATT  432

Query  181  GGCTATTATGCCCTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTTCCTCGTTC  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  431  GGCTATTATGCCCTTCGTCCCCTGCAACAGAACTTTACGATCCGAAGACCTTCCTCGTTC  372

Query  241  ACGCGGCGGTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCC  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  371  ACGCGGCGGTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCC  312

Query  301  GTAGGAGTCTGGGCCGTGTCTCAGTCCCAATGTGGCCGTTCATCCTCTCAGACCGGCTAC  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  311  GTAGGAGTCTGGGCCGTGTCTCAGTCCCAATGTGGCCGTTCATCCTCTCAGACCGGCTAC  252

Query  361  TGATCGTCGCCTTGGTGCGCCGTTACCGTCACCAACCAGCTAATCAGATGGGGCCCATCT  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251  TGATCGTCGCCTTGGTGCGCCGTTACCGTCACCAACCAGCTAATCAGATGGGGCCCATCT  192

Query  421  CCAAGCGATAGCTAAAAGGTCCCTTTGGTAATACTGCCATGCATCAGTATTACAACATTC  480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  191  CCAAGCGATAGCTAAAAGGTCCCTTTGGTAATACTGCCATGCATCAGTATTACAACATTC  132

Query  481  GGTATTAGCACCCCTTTCGGAGTGTTGTCCCCATCTTGGAGGCAGGTTGCCTACGCGTTA  540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  131  GGTATTAGCACCCCTTTCGGAGTGTTGTCCCCATCTTGGAGGCAGGTTGCCTACGCGTTA  72

Query  541  CTCACCCGTTTGCCACTAAACCCTTACCGAAATAAGAGCCTCGTTCGACTTGCATGTGTT  600
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  71   CTCACCCGTTTGCCACTAAACCCTTACCGAAATAAGAGCCTCGTTCGACTTGCATGTGTT  12

Query  601  AAGCACGCCGC  611
           ||||||||||||
Sbjct  11   AAGCACGCCGC  1
```

III E Partial 16S ribosomal gene sequences (ICBD strain PF-3) aligned

```
Query  1    TGGATGCGGCATCTACCATGCAGTCGAACGAGGCTCTTATTTCCGTGGGGCTTAGTGGCA  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGGATGCGGCATCTACCATGCAGTCGAACGAGGCTCTTATTTCCGTGGGGCTTAGTGGCA  60

Query  61   ACGGGTGAATAACGCGTAAGCGACCTGCCTCCAAGATGGGGACAACACTCCGAAAGGGGT  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   ACGGGTGAATAACGCGTAAGCGACCTGCCTCCAAGATGGGGACAACACTCCGAAAGGGGT  120

Query  121  GCTAATACCGAATGTTGTAATACTGCTGCATGGCAGTATTACCAAAGGTGGCTTTTAGCT  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GCTAATACCGAATGTTGTAATACTGCTGCATGGCAGTATTACCAAAGGTGGCTTTTAGCT  180

Query  181  ATCGCTTGGAGATGGGCCTGCGTCTGATTAGCTGGTTGGTGACGGTAACGGCGCACCAAG  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  ATCGCTTGGAGATGGGCCTGCGTCTGATTAGCTGGTTGGTGACGGTAACGGCGCACCAAG  240

Query  241  GCGACCATCATTAGCCGGTCTGAGAAGATGAACGGCCACATTGGGACTGAGACACGGACC  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GCGACCATCATTAGCCGGTCTGAGAAGATGAACGGCCACATTGGGACTGAGACACGGACC  300

Query  301  ACACTCCTACGGGAGGCAGCAGTGGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGC  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ACACTCCTACGGGAGGCAGCAGTGGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGC  360

Query  361  AACGCCGCGTGAACAAGGAAGGTCTTCGGATCGTAAAGTTCTGTTGCAGGGGACGAACGG  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  AACGCCGCGTGAACAAGGAAGGTCTTCGGATCGTAAAGTTCTGTTGCAGGGGACGAACGG  420

Query  421  CACTATAGCCAATAAGTATAGTGAATGACGGTACCCTGTTAGAAAGCCACGGCTAACTAC  480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CACTATAGCCAATAAGTATAGTGAATGACGGTACCCTGTTAGAAAGCCACGGCTAACTAC  480

Query  481  GTGCCAGCAGCCGCGGTAATACGTAGGCGGCAAGCGTTGTCCGGAATCATTGGGCGTAAA  540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GTGCCAGCAGCCGCGGTAATACGTAGGCGGCAAGCGTTGTCCGGAATCATTGGGCGTAAA  540

Query  541  GGGAGCGCATGCGGAACATTAATCGGATCTTAAAAGTGCGGGGCTCAACCCCGTGATGGG  600
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GGGAGCGCATGCGGAACATTAATCGGATCTTAAAAGTGCGGGGCTCAACCCCGTGATGGG  600

Query  601  GTCCGAACTGAGGTTCTTGAGTGCAGGAGAGGAAAGCTGAATTCCCAGTGTACGTTAAAA  660
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  GTCCGAACTGAGGTTCTTGAGTGCAGGAGAGGAAAGCTGAATTCCCAGTGTACGTTAAAA  660
```

III F Partial 16S ribosomal gene sequences (ICBD strain MC-1) aligned

```
Query  14   ATGATTCCGGAC-ACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCC  72
           |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  379  ATGATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCC  320

Query  73   GTGGCTTTCTCTTACGGTACCGTCACGGCGTATGGGTATTGACCATACACCCGTTCGTCC  132
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319  GTGGCTTTCTCTTACGGTACCGTCACGGCGTATGGGTATTGACCATACACCCGTTCGTCC  260

Query  133  CATATAACAGAGCTTTACAACCCGAAGGCCGTCTTCACTCACGCGGCGTTGCTCCGTCAG  192
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  259  CATATAACAGAGCTTTACAACCCGAAGGCCGTCTTCACTCACGCGGCGTTGCTCCGTCAG  200

Query  193  GCTTTCGCCCATTGCGGAAGATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTC  252
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199  GCTTTCGCCCATTGCGGAAGATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTC  140

Query  253  TCAGTTCCAATGTGGCCGTTCATCCTCTCAGACCGGCTACTGATCATTGCCTTGGTGGGC  312
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139  TCAGTTCCAATGTGGCCGTTCATCCTCTCAGACCGGCTACTGATCATTGCCTTGGTGGGC  80

Query  313  CGTTACCCCTCCAACTAGCTAATCAGACGCAAACCCCTCTTCCGGCGATAGCATATTCAG  372
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   CGTTACCCCTCCAACTAGCTAATCAGACGCAAACCCCTCTTCCGGCGATAGCATATTCAG  20

Query  373  TGGCCATCTT  382
           ||||||||||
Sbjct  19   TGGCCATCTT  10
```

III G Partial 16S ribosomal gene sequences (ICBD strain MC-2) aligned

```
Query  1    GTGCCAACTTCGCCATGATTCCGGACACGCTTGCCACCTACGTATTACCGCGGCTGCTGG  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  499  GTGCCAACTTCGCCATGATTCCGGACACGCTTGCCACCTACGTATTACCGCGGCTGCTGG  440

Query  61   CACGTAGTTAGCCGTGGCTTTCTCTTACGGTACCGTCACGGCGTATGGGTATTGACCATA  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  439  CACGTAGTTAGCCGTGGCTTTCTCTTACGGTACCGTCACGGCGTATGGGTATTGACCATA  380

Query  121  CACCCGTTCGTCCCATATAACAGAGCTTTACAACCCGAAGGCCGTCTTCACTCACGCGGC  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  379  CACCCGTTCGTCCCATATAACAGAGCTTTACAACCCGAAGGCCGTCTTCACTCACGCGGC  320

Query  181  GTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACTGCTGCCTCCCGTAGGAG  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319  GTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCCACTGCTGCCTCCCGTAGGAG  260

Query  241  TCTGGACCGTGTCTCAGTTCCAATGTGGCCGTTCATCCTCTCAGACCGGCTACTGATCAT  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  259  TCTGGACCGTGTCTCAGTTCCAATGTGGCCGTTCATCCTCTCAGACCGGCTACTGATCAT  200

Query  301  TGCCTTGGTGGGCCGTTACCCCTCCAACTAGCTAATCAGACGCAAACCCCTCTTCCGGCG  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199  TGCCTTGGTGGGCCGTTACCCCTCCAACTAGCTAATCAGACGCAAACCCCTCTTCCGGCG  140

Query  361  ATAGCATATTCAGTGGCCATCTTTTCTTCTAAAGGTCATGCGGCCTTTAGACGTCATTCG  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139  ATAGCATATTCAGTGGCCATCTTTTCTTCTAAAGGTCATGCGGCCTTTAGACGTCATTCG  80

Query  421  GTGAGTCTTCAAAAGGCGGGGGTATGATTGGTGTGTAGTGGTGGGGGGGGTTTCCTGATT  480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   GTGAGTCTTCAAAAGGCGGGGGTATGATTGGTGTGTAGTGGTGGGGGGGGTTTCCTGATT  20

Query  481  AAAAGGTGCGGCTGGTTCA  499
           ||||||||||||||||||
Sbjct  19   AAAAGGTGCGGCTGGTTCA  1
```

III H Partial 16S ribosomal gene sequences (ICBD strain MC-3) aligned

```
Query  1    TTGTTCCTTTTTAAAAAAGTTGGGCCCTTGTAATATGCTATCGCCGGAAGATGGGTTTGC  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  431  TTGTTCCTTTTTAAAAAAGTTGGGCCCTTGTAATATGCTATCGCCGGAAGATGGGTTTGC  372

Query  61   GTCTGATTAGCTAGTTGGAGGGGAACGGCCCACCAAGGCAATGATCAGTAGCCGGTCTGA  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  371  GTCTGATTAGCTAGTTGGAGGGGAACGGCCCACCAAGGCAATGATCAGTAGCCGGTCTGA  312

Query  121  GAGGATGAACGGACACATTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  311  GAGGATGAACGGACACATTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT  252

Query  181  GGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGAAGACGGC  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251  GGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGAAGACGGC  192

Query  241  CTTCGGGTTGTAAAGCTCTGTTATATGGGACGAACGGGCGTATGGTCAATACCCATACGC  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  191  CTTCGGGTTGTAAAGCTCTGTTATATGGGACGAACGGGCGTATGGTCAATACCCATACGC  132

Query  301  CGTGACGGTACCGTAAGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  131  CGTGACGGTACCGTAAGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG  72

Query  361  TAGGTGGCAAGCGTTGTCCGGAATCATTGGGCGTAAAGGGCGCGCAGGCGGTTCGGTAAG  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  71   TAGGTGGCAAGCGTTGTCCGGAATCATTGGGCGTAAAGGGCGCGCAGGCGGTTCGGTAAG  12

Query  421  TCGGTCTTAAA  431
           ||||||||||||
Sbjct  11   TCGGTCTTAAA  1
```

REFERENCES

REFERENCES

Alwine, J. C., Kemp, D. J. and Stark, G. R., 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proceedings of the National Academy of Sciences, 74(12), 5350-5354.

Alzwiy, I., A., and Morris, P., C., 2007. A mutation in the *Arabidopsis MAP kinase kinase* 9 gene results in enhanced seedling stress tolerance, Plant Science, 173(3), 302-308.

American Society of Brewing Chemists, 1992. Methods of Analysis, 8th (revised) (edn.), Beer analysis, bitterness (International method), The Society: St. Paul, MN, pp-1-44.

Anderson, R., 2003. Microbes and the origins of porter: Brewing History. The journal of the Brewery History Society, 113, 27-30.

Andrews, J. M. and Axcell, B.C., 2003. Wort boiling - evaporating the myths of the past, Technical Quarterly Master Brewer Association of America, 40**,** 249-254.

Andrews, J., M. 2004. A review of progress in mash separation technology Technical Quarterly of Master Brewer Association of America, **41,** 45-49.

Andrews, J., M., H., Hancock, J., C., Ludford-Brooks, J., Murfin, I., J., Houldsworth, L. and Phillips, M., 2011. 125th Anniversary Review: Some Recent Engineering Advances in Brewing and Distilling, Journal of the Institute of Brewing, 117(1), 23-32.

Arnold Jr., L., J. and Nelson, N., C., 1993. Acridinium ester labelling and purification of nucleotide probes, U.S. Patent 5,185,439.

Arnold Jr., L., J. and Nelson, N., C., 1999. Hybridization protection assay, U.S. Patent 6,004,745.

Arnold Jr., L., J., Hammond, P., W., Wiese, W., A. and Nelson, N., 1989. Assay formats involving acridinium-ester-labelled DNA probes. Journal of Clinical Chemistry, 35(8), 1588-1594.

Arnold Jr., L., J., Reynolds, M., A. and Bhatt, R., S., 1997. Non-nucleotide linking reagents for nucleotide probes, U.S. Patent 5,696,251

Arnold Jr., L., J., Reynolds, M., A., Bhatt, R., S., 2000. Non-nucleotide linking reagents for nucleotide probes, U.S. Patent 6,031,091.

Asano, S., Iijima, K., Suzuki, K., Motoyama, Y., Ogata, T. and Kitagawa, Y., 2009. Rapid detection and identification of beer-spoilage lactic acid bacteria by micro-colony method, Journal of Bioscience and Bioengineering, 108**,** 124-129.

Asano, S., Suzuki, K., Kuriyama, H., Yamashita, H., Kitagawa Y., 2008. Application of multiplex PCR to the detection of beer spoilage bacteria, Journal of American Society of Brewing Chemists, **66**, 37-42.

Ausubel, F., M., 1990. Current Protocols in Molecular Biology, Vol. 1, Green publishing associates and Wiley-Interscience, New York.

Axcell, B., C., 2007. R and D in brewing: the importance of underpinning knowledge, Proceedings of European Brewing Convention, **31**, 173.

Back, W. and Stackebrandt, E., 1978. DNS/DNS-Homologie studien innerhalb der Gattung Pediococcus, Archiv für Mikrobiologie, 118, 79–85.

Back, W., 2005. Brewery. In: Back, W., (ed.). Colour Atlas and Handbook of Beverage Biology. Nürnberg; Germany: Fachverlag Hans Carl, Germany, pp. 10–112.

Back, W., 1981. Nachweis und Identifizierung Gram-negativer bierschädlicher Bakterien. Monatsschrift brauwissenschaft, **128,** 1358-1362.

Back, W., 1994. Secondary contamination in the filling area, Brauwelt International, 4, 326-328.

Back, W., 2003. Biofilme in der Brauerei und Getränkeindustrie – 15 Jahre Praxiserfahrung. *Brauwelt online*, **24/25,** 1-5.

Back, W., Bohak, I., Ehrmann, M., Ludwig, W. and Schleifer, K.H., 1996. Revival of the species *Lactobacillus lindneri* and the design of a species specific oligonucleotide probe, Journal of Systematic and Applied Microbiology, 19, 322-325.

Back, W., Breu, S. and Weigand, C., 1988. Infektionsursachen im jahre 1987, Brauwelt 178, 1358-1362.

Back, W., Leibhard, M. and Bohak, I., 1992. Flash pasteurization – membrane filtration. Comparative biological safety. Brauwelt International, 12, 42– 49.

Barney, M., Volgyi, A., Navarro, A. and Ryder, D., 2001. Riboprinting and 16S rRNA gene sequencing for identification of brewery *Pediococcus* isolates, Journal of Applied Environmental Microbiology, 67, 553– 560.

Becker, M., M., Dickson, V., C., Norman, N., C. and Matsuda, E., 1999. The Double Helix Is Dehydrated: Evidence from the Hydrolysis of Acridinium Ester- Labeled Probes, Nucleic Acids Research, **38**, 5603-5611.

Becker, M., M., Majlessi, M. and Brentano, S., T., 2008. Kits and reaction mixtures containing modified probe molecules, U.S. Patent 7,399,852.

Bertani, G., 1952. Studies on Lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia* coli. Journal of Bacteriology, 62:293-300.

Bohak, I., Thelen, K. and Beimfohr, C., 2006. Description of *Lactobacillus backi sp. nov.*, an obligate beer-spoiling bacterium. Monatsschr. Brau., **59**, 78–82.

Boulton, C. and Quain, D., 2006. Brewing Yeast and Fermentation, Blackwell Science, Oxford, pp. 1-644

Brecher, M. E., Hogan, J., J., Boothe, G., Kerr, A., McClannan, L., Jacobs, M., R. and Longiaru, M., 1994. Platelet bacterial contamination and the use of a chemiluminescence-linked universal bacterial ribosomal RNA gene probe. Transfusion, 34(9), 750-755.

Briggs, D., E., Boulton, C., A., Brookes, P., A. and Stevens, R., 2006. Yeast biology. In: Brewing Science and Practice, Boulton, C. and Quain, D., (eds.), Brewing Yeast and Fermentation, Blackwell Science, Oxford, pp. 363-400.

British Beer and Pub Association's Statistical Handbook, 2010, pp.1- 123

British Beer and Pub Association's Statistical Handbook, 2011, pp. 1-119

Broens, L., 2008. Norit's creative solutions to the challenges of sustainability in breweries. EBC Environmental Sustainability Symposium, Stratford-on-Avon, 2008.

Brown, R. C., Li, Z., Rutter, A. J., Mu, X., Weeks, O. H., Smith, K., and Weeks, I., 2009. Development and application of a novel acridinium ester for use as a chemiluminescent emitter in nucleic acid hybridisation assays using chemiluminescence quenching. Organic and Bimolecular Chemistry, 7(2), 386-394.

Bunker, H. J., 1955. The survival of pathogenic bacteria in beer. Proceedings of European Brewing Conventions. Baden-Baden, 330-341.

Bunthof, C. J., Bloemen, K., Breeuwer, P., Rombouts, F. M., and Abee, T., 2001. Flow cytometric assessment of viability of lactic acid bacteria. Journal of Applied and Environmental Microbiology, 67(5), 2326-2335.

The Barth report, 2011. Hops 2010/11. (e-book), Nuremberg: Meier, H., G., (ed), John Barth & Sohn GmbH & Co KG. Nuremberg, pp. 1-32, *Accessed online: http://www.barthhaasgroup.com/images/pdfs/barthreport_20102011_englisch.pdf*

Caldwell, J. M., Juvonen, R., Brown, J., and Breidt, F., 2013. *Pectinatus sottacetonis* sp. nov. isolated from commercial pickle spoilage tank. International Journal of Systematic and Evolutionary microbiology. IJSEM Papers in Press. Published April 19, 2013 as doi:*10.1099/ijs.0.047886-0*.

Campbell, I., 2003. Microbiological methods in brewing analysis. In Priest, F., G. Campbell, I., Brewing Microbiology, 3rd Kluwer Academic/ Plenum Publisher, pp 367-392.

Casey, G. P., 1996. Practical applications of pulsed field electrophoresis and yeast chromosome fingerprinting in brewing QA and R & D, Technical quarterly-Master Brewers Association of the Americas, 33(1), 1-10.

Chaban, B., Deneer, H., Dowgiert, T., Hymers, J. and Ziola, B., 2005. The flagellin gene and protein from the brewing spoilage bacteria *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis*, Canadian Journal of Microbiology, **51,** 863-874.

Chihib, N. E. and Tholozan, J. L., 1999. Effect of rapid cooling and acidic pH on cellular homeostasis of *Pectinatus frisingensis*, a strictly anaerobic beer-spoilage bacterium. International Journal of Food Microbiology, **48,** 191-202.

Chihib, N. E., Monnerat, L., Membré, J. M., & Tholozan, J. L., 1999). Nisin, temperature and pH effects on growth and viability of Pectinatus frisingensis, a Gram-negative, strictly anaerobic beer-spoilage bacterium, Journal of Applied Microbiology, *87*(3), 438-446.

Chowdhury, I., Watier, D. and Hornez, J. P., 1995. Variability in survival of *Pectinatus cerevisiiphilus*, strictly anaerobic bacteria, under different oxygen conditions. Anaerobe, 1995, **1,** 151-156.

Chowdhury, I., Watier, D., Leguerinel, I. and Hornez, J. P., 1997. Effect of *Pectinatus cerevisiiphilus* on *Saccharomyces cerevisiae* concerning its growth and alcohol production in wort medium. Journal of Food Microbiology, **14,** 265-272.

Clancy, E., Glynn, B., Reddington, K., Smith, T., and Barry, T., 2012. Culture confirmation of Listeria monocytogenes using tmRNA as a diagnostics target. Journal of Microbiological Methods, 88(3), 427.

Colosia, M., 2004. Acid cleaning: Your future to greater savings, higher productivity, increased safety, and a better environment. Technical quarterly-Master Brewers Association of the Americas, *41*(2), 111-114.

De Man, J. C., Rogosa, M., and Sharpe, M. E., 1960. A medium for the cultivation of lactobacilli. Journal of Applied Microbiology, 23(1), 130-135**.**

De Mesmaeker, A., Altmann, K.,H., Waldner, A., Wendeborn, S., 1995. Backbone modifications in oligonucleotide and peptide nucleic acid systems. Current Opinion in Structural Biology, 5, (3)343–355.

Deutscher, M., P., 2006. Degradation of RNA in bacteria: comparison of mRNA and stable RNA. Nucleic acids research, 34(2), 659-666.

Dhingra, K., Talpaz, M., Riggs, M. G., Eastman, P. S., Zipf, T., Ku, S., and Kurzrock, R., 1991. Hybridization protection assay: a rapid, sensitive, and specific method for detection of Philadelphia chromosome-positive leukemias. Blood, 77(2), 238-242.

DiMichele, L.J., Lewis, M.J., 1993. Rapid species-specific detection of lactic acid bacteria from beer using polymerase chain reaction. Journal of American Society of Brewing Chemists 51, 63–66.

Dirksen, J., 2003. Chlorine dioxide for the brewing industry. Technical quarterly Master Brewers Association of the Americas, 40(2), 111-113.

Dirksen, J., 2005. Monitoring and controlling microbial contamination in the beer filling area. Technical quarterly Master Brewers Association of the Americas, *42*(1), 39-44.

Dobson, C.M., Deneer, H., Lee, S., Hemmingsen, S., Glaze, S.and Ziola, B., 2002. Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer. International Journal of Systematics and Evoutionary Microbiology, **52**, 2003–2010.

Dodeigne, C., Thunus, L., and Lejeune, R., 2000. Chemiluminescence as diagnostic tool. A review. Talanta, 51(3), 415-439.

Donhauser, S. and Jacob, F., 1988. Warum zählt Bier zu den reinsten Lebensmitteln? brauwelt, 128, 1452–1461.

Dowhanick, T., M., 1994. Advances in yeast and contaminant determination: The future of the so called 'rapid' methods. Proceedings of J. De Clerck Chair VI, Leuven, 11– 15.

Dull, C. L., Karr, T. and M. Kawasaki., 1998. SMMP Medium for the Selective Isolation of *Megasphaera* and *Pectinatus* (ICM). Journal of American Society of Brewing Chemists, 212-124.

Dürr, P., 1983, Luftkeimindikation bierschädlicher Bakterien: Neue Methode mittels Luftkeimsammelgerät und Luftkeimindikator. Brauwelt, **123,** 1652-1655.

Eaton, B., 2003. An Overview of Brewing, In Handbook of Brewing, (eds.), Priest, F., G., Stewart, G., G., Boca Raton, Florida: CRC Press, Taylor and Francis Group, pp. 77-89.

Ehrenfeld, E.E., Scheld, J., Miller, S.A. and Carpenter, C.R., 1996. Use of ATP-bioluminescence in the brewing industry. Technical Quarterly of Master Brewer Association of America , 33 (1), 59-62.

Ehrmann, M. A., Preissler, P., Danne, M. and Vogel, R. F., 2010. *Lactobacillus paucivorans* sp. nov., isolated from a brewery environment. International Journal of Systematic and Evolutionary Microbiology, 60(10), 2353-2357**.**

Engelmann, U. and Weiss, N., 1985. *Megasphaera cerevisiae* sp. nov.: A new Gram-negative obligately anaerobic coccus isolated from spoiled beer. Journal of Systematic and Applied Microbiology, **6,** 287-290.

Esslinger, H.M and Narziss, Beer, Ullmann's Encyclopedia of Industrial Chemistry, Vol-5, Wiley-VCH Verlag Co., Weinheim, Germany, pp. 177-221

FAO, 2009. Agribusiness Handbook Barley Malt Beer. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, pp. 1-70. *Available through: http://www.fao.org/docrep/fao/012/i1003e/i1003e00.pdf.*

Flahaut, S., Tierny, Y., Watier, D., Hornez, J. P., and Jeanfils, J., 2000. Impact of thermal variations on biochemical and physiological traits in *Pectinatus* sp., International Journal of food microbiology, 55(1), 53-61.

Franken, L., Van de Riet, M., Schasteen, E. and Pringle, A., 2000. A quantitative test for evaluating the effectiveness of cleaning chemicals, Technical Quarterly Master Brewer Association of America, 37 (3), 341-344.

Freier, S., M. and Altmann, K., H., 1997. The ups and downs of Nucleic acid duplex stability: structure- stability on chemically- modified DNA: RNA duplexes. Nucleic Acids Research, **25 (22)**, 4429-4443.

Gares, S., L., Whiting., M., S., Ingledew, W., M. and Ziola, B., 1993. Detection and identification of *Pectinatus cerevisiiphilus* using surface-reactive monoclonal antibodies in a membrane filter-based fluoroimmunoassay. Journal of American Society of Brewing Chemists, 51, 158–163.

Gaub, R., 2008, The green power of membranes. EBC Environmental Sustainability Symposium, Stratford-on-Avon, 2008.

Gilliland, R., B., 1971. Yeast classification. Journal of Institute of Brewing, **77**: 276–284.

Gonzalez, J. M., Jurado, V., Laiz, L., Zimmermann, J., Hermosin, B. and Saiz-Jimenez, C., 2004. *Pectinatus portalensis* nov. sp., a relatively fast-growing, coccoidal, novel Pectinatus species isolated from a wastewater treatment plant. Antonie Van Leeuwenhoek, **86,** 241-247.

Goto, S., Takahashi, A., Kamisango, K., and Matsubara, K., 2002. Single-nucleotide polymorphism analysis by hybridization protection assay on solid support. Analytical Biochemistry, 307(1), 25-32.

Gouy, M., Guindon, S., and Gascuel, O., 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution, 27(2), 221-224.

Granato, P. A., and Franz, M. R., 1989. Evaluation of a prototype DNA probe test for the noncultural diagnosis of gonorrhea. Journal of Clinical Microbiology, 27(4), 632-635.

Gunkel J., Voetz, M. and Rath., F., 2002. Effect of the malting barley variety (*Hordeum vulgare* L.) on fermentability. Journal of Institute of Brewing, **108**:355–361.

Haakensen, M. and Ziola, B., 2008. Identification of novel horA-harbouring bacteria capable of spoiling beer. Canadian Journal of Microbiology, 54, 321–325.

Haakensen, M., Dobson, C. M., Deneer, H. and Ziola, B., 2008b. Real-time PCR detection of bacteria belonging to the Firmicutes phylum. Int. J. Food Microbiol. 125, pp. 236–241.

Hage, T. and Wold, K., 2003. Practical experiences on the combat of a major Pectinatus and Megasphaera infection with the help of TaqMan realtime-PCR. Proceedings 29th EBC Congress, Dublin, Nürnberg, Germany: Fachverlag Hans Carl., pp. 1145–1148.

Haikara, A. and Helander, I., 2006. *Pectinatus*, *Megasphaera* and *Zymophilus*. In Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer and E. Stackebrandt, E., M., (eds.), The Prokaryotes, Vol. 4, Springer-Verlag: New York, pp. 965-981.

Haikara, A. and Lounatmaa, K., 1987. Characterization of *Megasphaera* sp., a new anaerobic beer spoilage coccus. Proceedings of European Brewing Convention Congress, Madrid, IRL Press, Oxford, pp. 473-480.

Haikara, A., 1983. Immunological characterization of *Pectinatus cerevisiiphilus* strains. Journal of Applied Environmental Microbiology, 46, 1054– 1058.

Haikara, A., 1984. Beer spoilage organisms. Occurrence and detection with particular reference to a new genus *Pectinatus*. Ph.D. thesis, Technical Research Centre of Finland Publications: Espoo, Finland, pp. 1-47.

Haikara, A., 1985. Detection of *Pectinatus* contaminants in beer. Journal of American Society of Brewing Chemists, 43, 43-46.

Haikara, A., 1991. The genera *Pectinatus* and *Megasphaera*. In, Balows, G., H., Trüper, M., Dwokin, W., Harder and S. H. Schleifer (eds.), The Prokaryotes, 2nd edn. Springer-Verlag: New York, pp. 1993-2004.

Haikara, A., Enari, T., M. and Lounatmaa, K., 1981. The genus *Pectinatus*, a new group of anaerobic beer spoilage bacteria. Proceedings of European Brewing Convention Congress, Copenhagen, IRL Press: Oxford, pp. 229-240.

Haikara, A., Mattila-Sandholm, T. and Manninen, M., 1990. Rapid detection of contaminants in pitching yeast using automated turbidometry. Journal of American Society of Brewing Chemists, 48, 92-95.

Haikara, A., Penttilä, L., Enari, T., M. and Lounatmaa, K., 1981. Microbiological, biochemical and electron microscopic characterization of a *Pectinatus* strain. Journal of Applied Environmental Microbiology, 41**,** 511-517.

Hakalehto, E., 2000. Characterization of *Pectinatus cerevisiiphilus* and *P. frisingensis* surface components. Use of synthetic peptides in the detection of some Gram-negative bacteria. Ph.D. Thesis, Natural and Environmental Sciences, Kuopio University Publications, Kuopio, Finland.

Harper, M. and Johnson, R., 1990. The predictive value of culture for diagnosis of gonorrhoea and Chlamydia infections. Journal Clinical Microbiology Newsletters, 12, 54-56.

Helander, I. M., Haikara, A., Sadovskaya, I., Vinogradov, E. and Salkinoja-Salonen, M. S., 2004. Lipopolysaccharides of anaerobic beer spoilage

bacteria of the genus *Pectinatus*–lipopolysaccharides of a Gram-positive genus. FEMS microbiology reviews, 28(5), 543-55.

Henegariu, O., Heerema, N., A., Dlouhy, S., R., Vance, G. H. and Vogt, P., H., 1997.Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques, 23(3), 504-511.

Hill, A. E., 2009. Microbiological stability of beer. In, Bamforth C., (ed.), Beer: A Quality Perspective, Academic Press, pp.163-184.

Hogan, J., J., 2000. Nucleic acid probes to *Listeria monocytogenes*. U.S. Patent 6,028,187.

Holah, J., T., 1992. Industrial monitoring: hygiene in food processing. In: Melo, L.,F., Bott, T.,R., Fletcher, M. and Capdeville, B., (eds.), Biofilms – Science and Technology. Kluwer Academic Publications, Dordrecht. pp. 645–659.

Hollerova´, I., Kubizniakova´, P., 2001. Monitoring Gram positive bacterial contamination in Czech breweries. Journal of Institute of Brewing, 107, 355– 358.

Ijima, K., Shizuka, A., Koji, S., Ogata, T., Kitagawa, Y., 2008. Modified Multiplex PCR method for Comprehensive detection of *Pectinatus* and Beer spoilage cocci. Bioscience Biotechnology Biochemistry, 72 (10), 2764-2766.

Janda, J., M. and Abbott, S., L., 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. Journal of Clinical Microbiology, 45(9), 2761-2764.

Jentsch, M., 2007. Top-fermented beer specialities in focus. Brauwelt International, **5**: 332–334.

Jespersen, L. and Jakobsen, M., 1996. Specific spoilage organisms in breweries and laboratory media for their detection. International Journal of Food Microbiology, 33, 139–155.

Jespersen, L., Lassen, S., and Jakobsen, M., 1993. Flow cytometric detection of wild yeast in lager breweries. International Journal of food microbiology, 17(4), 321-328.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S. and Madden, T. L., 2008. NCBI BLAST: a better web interface. Nucleic Acids Research , 36(2), W5-W9.

Juvonen, R. and Suihko, M. L., 2006. *Megasphaera paucivorans* sp. nov., *Megasphaera sueciensis* sp. nov. and *Pectinatus haikarae* sp. nov., isolated from brewery samples, and emended description of the genus *Pectinatu*s. International Journal of Systematic Evolutionary Microbiology, 56, 695-702.

Juvonen, R., 2009. DNA-based detection and characterisation of strictly anaerobic beer-spoilage bacteria. VTT Publications 723, Helsinki, Finland, pp 1-140

Juvonen, R., Koivula, T. and Haikara, A., 2008. Group-specific PCRRFLP and real-time PCR methods for detection and tentative discrimination of strictly anaerobic beer-spoilage bacteria of the class *Clostridia*. International Journal Food Microbiology, 125, 162-169.

Juvonen, R., Satokari, R., 1999. Detection of spoilage bacteria in beer by polymerase chain reaction. Journal of American Society of Brewing Chemists, 57, 99-103.

Juvonen, R., Satokari, R., Mallison, K. and Haikara, A., 1999. Detection of spoilage bacteria in beer by polymerase chain reaction. Journal of American Society of Brewing Chemists, **57,** 99-103.

Katz, S. H. and Maytag, F., 1991. Brewing an ancient beer. Archaeology, 44(4), 24-33.

Key Notes, 2008, Market report, 2008: Breweries and beer market, Worth S., (ed.) 27th (edn.), Key note publication, Hampton, UK, pp-1-95.

Key Notes, 2012, Market report, 2012: (executive summary) Breweries and beer market, Worth S., (ed.), Key note publication, Hampton, UK, accessed online *https://www.keynote.co.uk/market-intelligence/view/product/10566/breweries-%26-the-beer-market*.

Kiehne, M., Grönewald, C. and Chevalier, F., 2005. Detection and identification of beer spoilage bacteria using real-time polymerase chain reaction. Technical Quarterly Master Brewers Association of America, **42**, 214–218.

Kim, Y. J., Teletia, N., Ruotti, V., Maher, C. A., Chinnaiyan, A. M., Stewart, R., Patel, J. M., 2009. Probe-Match: a tool for aligning oligonucleotide sequences. Bioinformatics, 25 (11), 1424-1425.

Kindraka, J. A., 1987. Evaluation of NBB anaerobic medium for beer spoilage organisms. Technical Quarterly Master Brewers Association of America, 24, 146-15.

Kirchner, G., Lurz, R., Matsuzawa, K., 1980. Biertru¨bungen durch Bakterien der Gattung Bacteroides. Mon.schr. Brau. 33, 461– 467.

Kirin Institute of Food and Lifestyle report, 2012. Global Beer Production by Country in 2011, Vol. 36, (Press release), August 8, 2012. Accessed online: *http://www.kirinholdings.co.jp/english/news/2012/0808_01.html*

Kloos, W., E., Tornabene, T., G. and Schleifer, K., H., 1974. Isolation and characterization of micrococci from human skin, including two new species: Micrococcus lylae and *Micrococcus kristinae*. International Journal of Systematic Bacteriology, 24(1), 79-101.

Koivula, T., Juvonen, R., Haikara, A. and Suihko, M., L., 2006. Characterization of the brewery spoilage bacterium *Obesumbacterium proteus* by automated ribotyping and development of PCR methods for its biotype 1. Journal of Applied Microbiology, 100, 398–406.

Kozulis, J., A. and Page, H., E., 1968. A new universal beer agar medium for enumeration of wort and beer microorganisms. Proceedings of American Society of Brewing Chemists, 68, 52-8.

Kunze, W., 1996. Technology Brewing and Malting. Wainwright T, translator, VLB Berlin, Germany.

Lamond, A. I., and Sproat, B. S., 1993. Antisense oligonucleotides made of 2'- O-alkyl RNA: their properties and applications in RNA biochemistry. FEBS letters, 325(1), 123-127.

Lappalainen, J., Loikkanen, S., Havana, M., Karp, M., Sjoberg, A. M. and Wietanin, G., 2000. Microbial testing methods for detection of residual cleaning agents and disinfectants - prevention of ATP bioluminescence measurements in the food industry. Journal of Food Protection, 63, 210-215.

Larson, E. L., and Morton, H., E., 1991. Alcohols, In: S., S., Block (ed.), disinfection, sterilization, and preservation, 4th ed., Lea and Febiger, Philadelphia, Pa. pp. 191–203

Lawrence, D.R., 1988. Spoilage organisms in beer. In: Robinson, R., K., (Ed.), Developments in Food Microbiology. Elsevier, London, pp. 1 – 48.

Lee, S.Y., 1994. SMMP- a medium for selective isolation for *Megasphaera* and *Pectinatus* from the brewery. Journal of American Society of Brewing Chemists, 52, 115– 119.

Lee, S.,Y., Mabee, M.,S., Jangaard, N., O., 1978. *Pectinatus,* a new genus of the family bacteriodaceae. International Journal of Systematic Bacteriology, 28, 582– 594.

Lee, S., Y., Mabee, M., S., Jangaard, N., O. and Horiuchi, E., K., 1980. *Pectinatus*, a new genus of bacteria capable of growth in hopped beer. Journal of Institute of Brewing 86, 28–30.

Lewis, M., J. and Young, T., W., 2002. Brewing, 2nd, (edn.), Kluwer academic/ Springer Verlag, New York, pp 1-398.

Lindholm, L., Sarkkinen, H., 2004. Direct identification of gram-positive cocci from routine blood cultures by using AccuProbe tests. Journal of Clinical Microbiology, 42(12), 5609-5613.

Livezey, K., Kaplan, S., Bubeck-Barrett, A., Wisniewski, M. and Becker, M. M., 2013. A New Generation of Foodborne Pathogen Detection Based on Ribosomal RNA. Annual Reviews in Food Science and Technology, 4, 313-325.

Lodolo, E. J., Kock, J. L., Axcell, B. C., and Brooks, M., 2008. The yeast Saccharomyces cerevisiae–the main character in beer brewing. FEMS yeast research, 8(7), 1018-1036.

Mackay, I. M., 2004. Real-time PCR in the microbiology laboratory. Journal of Clinical Microbiology and Infection, 10(3), 190-212.

Mackay, I., M., Mackay, J. F., Nissen, M., D. and Sloots, T., P., 2007. Real-time PCR: history and fluorogenic chemistries. In: Mackay, I. M (ed.), Real-Time PCR in Microbiology. Norfolk, UK: Caister Academic Press. pp. 1–39.

Majlessi, M. and Becker, M., M., 2008. Formation of the double helix: a mutational study. Nucleic Acids Research, 36:9, 2981–2989.

Mansfield, E., S., Worley, J., M., McKenzie, S., E., Surrey, S., Rappaport, E. and Fortina, P., 1995. Nucleic acid detection using non-radioactive labelling methods. Molecular and Cellular probes, 9(3), 145-156.

March, C., Manclus, J. J., Abad, A., Navarro, A. and Montoya, A., 2005. Rapid detection and counting of viable beer-spoilage lactic acid bacteria using a monoclonal chemiluminescence enzyme immunoassay and a CCD camera. Journal of Immunological Methods, 303, 92–104.

Marchandin, H., Jumas-Bilak, E., Gay, B., Teyssier, C., Jean-Pierre, H., Siméon de Buochberg, M., Carrière, C. and Carlier, J., P., 2003. Phylogenetic analysis of some Sporomusa sub-branch members isolated from human clinical specimens: description of *Megasphaera micronuciformis* sp. nov. International Journal of Systematic and Evolutionary Microbiology, 53, pp. 547–553.

Marchandin, H.; Teyssier, C.; Campos, J.; Jean-Pierre, H.; Roger, F.; Gay, B.; Carlier, J. -P.; Jumas-Bilak, E., 2009. *Negativicoccus succinicivorans* gen. Nov., sp.

Nov., isolated from human clinical samples, emended description of the family Veillonellaceae and description of *Negativicutes* classis nov., *Selenomonadales* ord. nov. and *Acidaminococcaceae* fam. nov. In the bacterial phylum, *Firmicutes*. International Journal of Systematic and Evolutionary Microbiology **60** (6): 1271–1279.

Marlowe, E. M., Hogan, J. J., Hindler, J. F., Andruszkiewicz, I., Gordon, P., Bruckner, D. A., 2003. Application of an rRNA probe matrix for rapid identification of bacteria and fungi from routine blood culture, Journal of Clinical Microbiology, 41(11), 5127-5133.

Matoulková, D., Kosar, K., Sigler, K., 2012. Rapid, Simple, and Specific Cultivation-Based Method for Detection of *Pectinatus* spp. in Brewery Samples. Journal of American Society of Brewing Chemists, 70(1), 29.

M**azumdar, A., Majlessi, M. and Becker, M., M.,** 1998. A high throughput method to investigate oligonucleotide hybridization kinetics and thermodynamics. Nucleic Acids Research, 26:8, 1996-2000.

McCapra, F. and Beheshti, I., 1985. Selected chemical reactions that produce light, (Vol-1), CRC Press, Taylor and Francis Group, Boca Raton, Florida. pp. 9-42.

McCapra, F. and Richardson, D., G., 1964. The mechanism of chemiluminescence: a new chemiluminescent reaction. Tetrahedron Letters, 5(43), 3167-3172.

McCapra, F., 1970. Chemiluminescence of organic compounds. Journal of Pure and Applied. Chemistry, 24(61), 1-629.

McCapra, F., 1976. Chemical mechanisms in bio-chemiluminescence. Accounts of Chemical Research, 9, 201-108.

McDonnell, G. and Russell, A. D., 1999. Antiseptics and disinfectants: activity, action, and resistance, Clinical microbiology reviews, 12(1), 147-179.

McElevey, C. A., 1974. A modern mash filter. Technical Quarterly of Master Brewer Association of America, 11, 233-239.

Meier, H., Amann, R., Ludwig, W. and Schleifer, K. H., 1999. Specific oligonucleotide probes for *in situ* detection of a major group of gram-positive bacteria with low DNA G+C content. Journal of Systematic and Applied Microbiology, 22, 186–196.

Meilgaard, M. C., 1975. Flavor chemistry of Beer: Part II: Flavor and threshold of 239 aroma volatiles. Tech. Quarterly Master Brewer Association of America, 1975, 12, 151-168.

Melamed, M., R., T., Lindmo, and Mendelsohn, M., L., 1990. Flow cytometry and sorting, 2nd ed. Wiley-Liss Inc., New York.

Melis, M., 1993. The 2001 mash filter – practical experience. Brauwelt International, 120-122.

Meng, S., Wang, D., Liu, X., Zhang, Y., Guo, L., Lin, Z. and Pavlovic, M., 2012. Rapid Detection of *Lactobacillus brevis* in Beer Production by the Combination of Microcolony and Fluorescence In Situ Hybridization (FISH).Journal of the American Society of Brewing Chemists, 70(2), 91-94.

Menz, G., Aldred, P. and Vriesekoop, F., 2009. Pathogens in beer. In: V.R. Preedy, (ed), In Beer in Health and Disease Prevention, Academic Press: Amsterdam, pp 403-413.

Menz, G., Vriesekoop, F., Zarei, M., Zhu, B. and Aldred, P., 2010. The growth and survival of food-borne pathogens in sweet and fermenting brewers' wort. International Journal of Food Microbiology, 140(1), 19-25.

Moore, L., V., H., Bourne, D., M. and Moore, W., E., C., 1994. Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic Gram negative bacilli. International Journal of Systematic and Bacteriology, 44, 338–347.

Motoyama, Y. and Ogata, T., 2000. Detection of *Pectinatus* spp. by PCR using 16S-23S rDNA spacer regions. Journal of American Society of Brewing Chemists, **58,** 4-7.

Motoyama, Y., 2003. The study on strictly anaerobic bacteria capable of spoiling beer. Ph.D. thesis, Tokyo University: Tokyo, Japan.

Motoyama, Y., Ogata, T. and Sakai, K., 1998. Characterization of *Pectinatus cerevisiiphilus* and *P. frisingensis* by ribotyping, Journal of American Society of Brewing Chemists, **56,** 19-23.

Mozola, M., A., 2000. Detection of Microorganisms in Foods using DNA probes targeted to Ribosomal RNA sequences, Food Biotechnology, 14(3), 173- 194.

Mullis, K. B., Faloona, F. A., Scharf, S. J., Saiki, R. K., Horn, G. T. and Erlich, H. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. In Cold Spring Harbour Symposia on Quantative Biology, 51(1) pp. 263-273.

Mullis, K., B., Faloona, F., A., 1987. Specific synthesis of DNA invitro via Polymerase chain reaction, Methods in Enzymology, 155, 335-350.

Nakagawa, A., 1978. Beer-spoilage lactic acid bacteria – Principal characteristics and simple methods of their selective detection. Bulletin of Brewing Science, 1978, **24,** 1-10.

Natrajan, A. A., Sells, T., Schroeder, H., Yang, G., Sharpe, D., Jiang, Q., Lukinsky, H. and Law, S. J., 2011. Applications of acridinium compounds and derivatives in homogeneous assays. U.S. Patent No. 7,875,467.

Natrajan, A., and Sharpe, D., 2013. Synthesis and properties of differently charged chemiluminescent acridinium ester labels. Organic and Biomolecular Chemistry, 11(6), 1026-1039.

Nelson, N., C. and McDonough, S., H., 1990. Application of Hybridisation Protection Assay (HPA) to PCR. In Mullis, K., B., Ferre, F. Gibbs, R., A., Birkhauser (Eds), The Polymerase Chain Reaction, Boston, Massachusetts, pp 151-163.

Nelson, N., C., 1998. Rapid detection of genetic mutations using the chemiluminescent hybridization protection assay (HPA): overview and comparison with other methods. Critical Reviews in Clinical Laboratory Science, 35 (5), 369-414.

Nelson, N., C., Cheikh, A., B., Matsuda, E. and Becker, M., M., 1996. Simultaneous detection of multiple nucleic acid targets in a homogeneous format. Biochemistry, 35(25), 8429-8438.

Nelson, N., C., Kacian, D., L., 1990. Chemiluminescent DNA probes: a comparison of the acridinium ester and dioxetane detection systems and their use in clinical diagnostic assays. Clinica Chimica Acta, 194 (1), 73-90.

Nelson, N., C., Reynolds, M., A., Arnold Jr., L., J., 1995. Detection of acridinium esters by chemiluminescence. In: Kricka, L., J., (Ed), Nonisotopic probing, blotting, and sequencing. Academic Press, San Diego, California. pp. 391-428.

Ninet, B., Bannerman, E. and Bille, J., 1993. Assessment of the Accuprobe *Listeria monocytogens* culture identification reagent kit for rapid colony confirmation and its application in various enrichment broths. Journal of Applied Environmental Microbiology, **58**, 4055-4059.

Odebrecht, E., Schmidt, H., J. Franco, B., G., M., 2000. Studies on applicability of bioluminescence in the brewery. Comparative studies and critical evaluation, Brauwelt, 1904–1905.

Oerther, D., B., de los Reyes, F., L., 2001. Molecular methods in biological systems, Water Environmental Research, 73(5), 1-35.

Ohnishi, A., Abe, S., Nashirozawa, S., Shimada, S., Fujimoto, N., and Suzuki, M., 2011. Development of a 16S rRNA gene primer and PCR-restriction fragment length polymorphism method for rapid detection of members of the genus *Megasphaera* and species-level identification, Journal of Applied and environmental microbiology, 77(15), 5533-5535.

Partis, L., Newton, K., Murby, J. and Wells, R., J., 1994. Inhibitory effects of enrichment media on the Accuprobe test for Listeria monocytogens. Journal of Applied and Environmental Microbiology, 58, 1693-1694.

Pfannebecker, J., and Fröhlich, J., 2008. Use of a species-specific multiplex PCR for the identification of *Pediococci*. International Journal of food microbiology, 128(2), 288-296.

Plamer, G., H., 2003. Barley and Malt, In Handbook of Brewing, (eds.), Priest, F., G., Stewart, G., G., Boca Raton, Florida: CRC Press, Taylor and Francis Group. pp 139-160.

Polaina, J., 2002. Brewer's yeast: genetics and biotechnology. Applied Mycology and Biotechnology, 2, 1-17.

Powell, C. D., Quain, D. E. and Smart, K. A. (2003). The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation. FEMS yeast research, 3(2), 149-157.

Praeckel U., 2009. Cleaning and Disinfecting. In: Handbook of Brewing: Process, Technology, Markets (ed.) Eßlinger, H., M., Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. 477-490.

Pratt, P., L., Bryce, J. H. and Stewart, G., G., 2003. The effects of osmotic pressure and ethanol on yeast viability and morphology. Journal of the Institute of Brewing, 109(3), 218-228.

Priest, F. G., and Campbell, I., (eds.) 2003. Brewing microbiology (3rd edn.), Kluwer Academic/ Plenum Publisher, New York, pp-1-399.

Quain, D., 2008. Coming 'ome - the return of yeast genetics. Brewer and Distiller International, 34-36.

Quain, D., E., 2006. The yeast 'supply chain'. Brewer and Distiller 2, 19–23.

Quain, D., Smith, I., 2009. The long and short of maturation - the Brewers' Guardian, 56-61.

Rainbow, C., 1981. Beer spoilage microorganisms. In: Pollock, J., R., A. (Ed), Brewing Science Vol. 2, Academic Press: New York, pp. 491-550.

Rainieri S, K., Kaneko, Y., Mikata, K., Nakao, Y. and Ashikari, T., 2006. Pure and mixed genetic lines of *Saccharomyces bayanus* and *Saccharomyces pastorianus* and their contribution to the lager brewing strain genome. Journal of Applied Environmental Microbiology, **72**, 3968–3974.

Rank, G., H., Casey, G., and Xiao, W., 1988. Gene transfer in Industrial *Saccharomyces* yeast. Food Biotechnology, **2**: 1–41.

Razavi, Z. and McCapra, F., 2000. Stable and versatile active acridinium esters. Luminescence, 15(4), 239-244.

Roda, A. and Guardigli, M., 2012. Analytical chemiluminescence and bioluminescence: latest achievements and new horizons. Journal of Analytical and Bioanalytical Chemistry, 402(1), 69-76.

Rogosa, M., 1971**.** Transfer of *Peptostreptococcus elsdenii* Gutierrez et al. to a new genus, Megasphaera M. elsdenii (Gutierrez et al.) comb. nov. International Journal of Systematic Bacteriology, 21, 187–189.

Russell, I. and Stewart, R., 2003. Rapid detection and identification of microbial spoilage, In: Priest, F., G. and Campbell, I., (eds.), Brewing Microbiology. 3rd edition, Kluywer Academic/ Plenum Publisher, New York, pp 267-307.

Sakamoto, K., and Konings, W. N., 2003. Beer spoilage bacteria and hop resistance. International Journal of food microbiology, 89(2), 105-124.

Sakamoto, K., Funahashi, W., Yamashita, H. and Masakazu, E., 1997. A reliable method for detection and identification of beer-spoilage bacteria with internal positive control PCR (IPC-PCR). Proceedings of the 26th European Brewery Convention Congress, Maastricht. New York, USA: Oxford University Press, Inc. pp. 631–638.

Sambrook, J. and Russell, D., W., 2001. Molecular cloning: a laboratory manual 3rd (ed.) Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory.

Sami, M., Yamashita, H., Kadokura, H., Yoda, K. and Yamasaki, M. 1997. A new and rapid method for determination of beer-spoilage ability of *lactobacilli*. Journal of American Society of Brewing Chemists, 55, pp. 137–140.

Satokari, R., Juvonen, R., Mallison, K., von Wright, A. and Haikara, A., 1998. Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by polymerase chain reaction and colorimetric microplate hybridization. International Journal Food Microbiology, **45,** 119-127.

Schleifer, K., H., Leuteritz, M., Weiss, N., Ludwig, W., Kirchhof, G. and Seidel, R., H., 1990. Taxonomic study of anaerobic, Gram-negative, rod-shaped bacteria from breweries: emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingensis* sp. nov., *Selenomonas lacticifex* sp. nov., *Zymophilus raffinosivorans* gen. nov., sp. nov., and *Zymophilus paucivorans* sp. nov. International Journal of Systematic and Evolutionary Microbiology, **40,** 19-27.

Seidel, R., H., 1990. und andere morphologisch ähnliche Gram-negative, anaerobe Stäbchen aus dem Brauereibereich. Monatsschrift für brauwissenschaft, **43,** 101-105.

Seidel, R., H., Back, W., and Weiss, H., 1979. Isolation and systemic classification of beer spoilage, Gram-negative bacteria: Part III. What dangers do the Gram-negative cocci and rods described in parts I and II represent for beer?, Brauwissenschaft, 32 , 262–270.

Shama, G., and Malik, D., J., 2012. The uses and abuses of rapid bioluminescence-based ATP assays. International Journal of Hygiene and Environmental Health, 216 (2), 115–125.

Shapiro, H., M., 1995. Practical flow cytometry, Liss, A., R., (ed.), 3rd (Edn), Inc., New York.

Sicard, D., and Legras, J., L., 2011. Bread, beer and wine: Yeast domestication in the *Saccharomyces sensu strict* complex. Comptes rendus biologies, 334(3), 229-236.

Siebert, K., J., 1999. Modeling the flavor thresholds of organic acids in beer as a function of their molecular properties, Food quality and preference, *10*(2), 129-137.

Simpson, W., J. and Smith, A., R., W., 1992. Factors affecting antibacterial activity of hop compounds and their derivatives. Journal of Applied Bacteriology, 72, 327–334.

Simpson, W.J., 1993. Ionophoric action of iso trans-humulone on *Lactobacillus brevis*. Journal of General Microbiology, 139, 1041–1045.

Soberka, R., Sciazko, D. and Warzecha, A., 1988, *Pectinatus* nouvelle bactérie pouvant affecter la stabilité biologique du moüt et de la biére. Bioscience, **19,** 31-37.

Southern, E., M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis, Journal of molecular biology, 98(3), 503-517.

Stahl, D., A., and Amann. R., 1991. Development and application of nucleic acid probes, In: E., Stackebrandt and M., Goodfellow (eds.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, Inc., New York. pp. 205-248.

Stewart, J., S., Dowhanick, T., M., 1996. Rapid detection of lactic acid bacteria in fermenter samples using a nested polymerase chain reaction. Journal of American Society of Brewing Chemists, 54, 78–84.

Storgårds, E., 2000. Process hygiene control in beer production and dispensing, (Academic Dissertation), VTT Publication 410, Helsinki, Finland, pp. 1-108.

Storgårds, E., Tapani, K., Hartwall, P., Saleva, R. and Suihko, M., L., 2006. Microbial attachment and biofilm formation in brewery bottling plants. Journal of American Society of Brewing Chemists, 64, 8-15.

Strauss, C., J., 2005. The role of lipids in the flocculation of *Saccharomyces cerevisiae*. PhD thesis, University of the Free State, Bloemfontein, South Africa. pp. 1-114.

Strömpl, C., Tindall, B., J., Jarvis, G., N., Lünsdorf, H., Moore, E. R. and Hippe, H., 1999. A re-evaluation of the taxonomy of the genus *Anaerovibrio*, with the reclassification of *Anaerovibrio glycerini* as *Anaerosinus glycerini* gen. nov., comb. nov., and *Anaerovibrio burkinabensis* as *Anaeroarcus burkinensi*s [corrig.] gen. nov., comb. nov. International Journal of Systematic and Evolutionary Microbiology, 49(4), 1861-1872.

Suihko, M. L., and Haikara, A., 1990. Maintenance of the anaerobic beer spoilage bacteria *Pectinatus* and *Megasphaera*, Food microbiology, 7(1), 33-41.

Suzuki, K., 2011. 125th Anniversary Review: Microbiological instability of beer caused by spoilage bacteria. Journal of the Institute of Brewing, 117(2), 131-155.

Suzuki, K., Asano, S., Iijima, K. and Kitamoto, K., 2008. Sake and beer spoilage lactic acid bacteria – A review. Journal of Institute of Brewing, **114,** 209-223.

Suzuki, K., Asano, S., Iijima, K., Kuriyama, H., & Kitagawa, Y., 2008a. Development of detection medium for hard-to-culture beer-spoilage lactic acid bacteria. Journal of applied microbiology, 104(5), 1458-1470.

Suzuki, K., Funahashi, W., Koyanagi, M. and Yamashita, H., 2004. *Lactobacillus paracollinoides* sp. nov., isolated from brewery environments. International Journal of Systematic and Evolutionary Microbiology, 54, 115-117.

Suzuki, K., Iijima, K., Ozaki, K. and Yamashita, H., 2005. Isolation of hop-sensitive variant from *Lactobacillus lindneri* and identification of genetic marker for beer spoilage ability of lactic acid bacteria. Journal of Applied Environmental Microbiology, 71, 5089-5097.

Suzuki, K., Iijima, K., Sakamoto, K., Sami, M. and Yamashita H., 2006. A review of hop resistance in beer spoilage lactic acid bacteria. Journal of The Institute of Brewing, **112,** 173-191.

Taguchi, H., Ohkochi, M., Uehara, H., Kojima, K. and Mawatari, M., 1990. KOT medium, a new medium for the detection of beer spoilage lactic acid bacteria. Journal of American Society of Brewing Chemists, **48,** 72-75.

Taguchi, H., Yasui, T. and Kamiya, T. , 1995. An improved discrimination method of beer spoilage lactobacilli from non-spoilage brewery contaminants. European Brewery Convention, Proceedings of the 25th Congress, Brussels. New York, USA: Oxford University Press, pp. 621–628.

Takahashi, N., 1983. Presumed *Pectinatus* strain isolated from Japanese beer. Bulletin Brewing Science, **28,** 11-14.

Takahashi, T., Nakakita, Y., Sugiyama, H., Shigyo, T. and Shinotsuka, K.,1999. Classification and identification of strains of *Lactobacillus brevis* based on electrophoretic characterization of d-lactate dehydrogenase: Relationship between d-lactate dehydrogenase and beer-spoilage ability, Journal of bioscience and bioengineering, 88(5), 500-506.

Tenover, F., C., Carlson, L., Barbagallo, S. and Nachamkin, I., 1990. DNA probe culture confirmation assay for identification of thermophilic *Campylobacter* species. Journal of Clinical Microbiology, 28(6), 1284-1287.

Teunissen, A., Steensma, H., Y., 1995. Review: the dominant flocculation genes of *Saccharomyces cerevisiae*. Yeast, **11**:1001–1013.

Tholozan, J,. L. and Jacquemont, J., 1999. Physiological response of *Pectinatus frisingensis*, a beer spoilage bacterium, to mild heat treatments. Canadian Journal of Microbiology, **45,** 598-606.

Tholozan, J. L., Membre, J. M. and Grivet, J. P., 1997. Physiology and development of *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis*, two strict anaerobic beer spoilage bacteria. International Journal of Food Microbiology, **35,** 29-39.

Tholozan, J., L., Membré, J., M. and Kubaczka, M., 1996. Effects of culture conditions on *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis* metabolism: a physiological and statistical approach. Journal of Applied Bacteriology, **80,** 418-424.

Timke, M., Wolking, D., Wang-Lieu, W. Q., Altendorf, K. and Lipski, K., 2005. Microbial composition of biofilms in a brewery investigated by fatty acid analysis, fluorescence in situ hybridisation and isolation techniques. Journal of Applied Microbiology and Biotechnology, 66**,** 100-107.

Tsuchiya, Y., 2002. Antibodies for the detection of beer spoilage lactic acid bacteria and kit employing the antibodies.U.S. Patent 6,395,500

Tsuchiya, Y., Kaneda, H., Kano, Y., and Koshino, S., 1992. Detection of beer spoilage organisms by polymerase chain reaction technology. Journal of the American Society of Brewing Chemists, 50(2), 64-67.

Tsuchiya, Y., Kano, Y., and Koshino, S., 1993. Detection of *Lactobacillus brevis* in beer using polymerase chain reaction technology. Journal of the American Society of Brewing Chemists, 51.

Van Nierop, S., N., E., Rautenbach, M., Axcell, B. C. and Cantrell, I. C., 2006. The impact of microorganisms on barley and malt quality-A review, Journal of American Society of Brewing Chemists, 64(2), 69.

Vaughan, A., O'Sullivan, T. and Van Sinderen, D., 2005. Enhancing the microbiological stability of malt and beer - a review. Journal of Institute of Brewing, **111**, 355–371.

Vaughan, M., A. and Martini, A., 1998. *Saccharomyces Meyen ex Reess.* In: Kurtzman, C., P., and Fell, J., W., (eds.), The Yeasts, 4th (edn.), Elsevier, Amsterdam, pp.358–371.

Vereecke, C. and Arahal, D., R., 2008. The status of the species *Pectinatus portalensis* Gonzalez et al. 2005, Request for an opinion. International Journal of Systematic and Evolutionary Microbiology, **58,** 1507.

Verstrepen, K., J., Derdelinckx, G., Verachtert., H. and Delvaux, F., R., 2003. Yeast flocculation, that brewers should know. Journal of Applied Microbiology and Biotechnology, **61**: 197–206.

Viscidi, R. P., Connelly, C. J. and Yolken, R., H., 1986. Novel chemical method for the preparation of nucleic acids for nonisotopic hybridization. Journal of Clinical Microbiology, 23(2), 311-317.

Vriesekoop, F., Krahl, M., Hucker, B. and Menz, G., 2013. 125th Anniversary Review: Bacteria in brewing: The good, the bad and the ugly. Journal of Institute of Brewing, 118 (4), 335-345.

Weber, D., G., Sahm, K., Polen,T., Wendisch, V., F. and Antranikian, G., 2008. Oligonucleotide microarray for detection of viable beer spoilage bacteria, Journal of Applied Microbiology, 105, 951-962.

Weeks, I., Beheshti, I., McCapra, F., Campbell, A. K. and Woodhead, J. S., 1983. Acridinium esters as high-specific-activity labels in immunoassay. Journal of Clinical Chemistry, 29(8), 1474-1479.

Weeks, K., M. and Crothers, D., M., 1993. Major groove accessibility of RNA. Science, 261, 1574-1574.

Wetmur, J., G., and Fresco, J., 1991. DNA probes: applications of the principles of nucleic acid hybridization. Critical Reviews in Biochemistry and Molecular Biology, 26 (3-4), 227-259.

Whiting, M., Crichlow, M., Ingledew, W. M. and Ziola, B., 1992. Detection of *Pediococcus* spp. in brewing yeast by a rapid immunoassay. Journal of Applied Environmental Microbiology, 58, 713–716.

Wightman, P., Quain, D., E. and Meaden, P., G., 1996. Analysis of production brewing strains of yeast by DNA fingerprinting, Letters in Applied Microbiology, 22, 85 – 89.

Willems, A. and Collins, M., D. 1995. Phylogenetic placement of *Dialister pneumosintes* (formerly *Bacteroides pneumosintes*) within the Sporomusa sub-branch of the *Clostridium* subphylum of the gram-positive bacteria. International Journal of Systematic Bacteriology, 45, 403–405.

Yasuhara, T., Yuuki, T. and Kamagi, N., 2001. Novel Quantitative method for detection of Pectinatus using rRNA targeted fluorescent probes. Journal of American Society of Brewing Chemists, 59 (3), 117-121.

Yasui, T. and Yoda, K., 1997. Purification and partial characterization of an antigen specific to *Lactobacillus brevis* strains with beer spoilage activity. FEMS Microbiology Letters, 151**,** 169-176.

Yasui, T., Okamoto, T., Taguchi, H., 1997. A specific oligonucleotide primer for the rapid detection of *Lactobacillus lindneri* by polymerase chain reaction. Canadian Journal of Microbiology, 43, 157– 163.

Yasui, T., Yoda, K. and Kamiya, T., 1995. Analysis of S-layer proteins of *Lactobacillus brevis*. FEMS Microbiology Letters, 131, 181– 186.

Zhang, W. W., Fang, M. X., Tan, H. Q., Zhang, X. Q., Wu, M. and Zhu, X. F., 2012. *Pectinatus brassicae sp. nov.,* a Gram-negative, anaerobic bacterium isolated from salty wastewater. International Journal of Systematic and Evolutionary Microbiology, 62(9), 2145-2149.

Zhao, L., Sun, L. and Chu, X., 2009. Chemiluminescence immunoassay. Trends in Analytical Chemistry, 28(4), 404-415.

Ziola, B., Gee, L., Berg, N.N. and Lee, S.Y., 2000a. Serogroup of the beer spoilage bacterium *Megasphaera cerevisiae* correlate with the molecular weight of the major EDTA-extractable surface protein. Canadian Journal of Microbiology, 46, 95-100.

Ziola, B., Ulmer, M., Bueckert, J. and Giesbrecht, D., 2000b. Monoclonal antibodies showing surface reactivity with *Lactobacillus* and *Pediococcus* beer spoilage bacteria. Journal of American Society of Brewing Chemists, 58, 63–68.

Zomer, B., Collé, L., Jedyńska, A., Pasterkamp, G., Kooter, I. and Bloemen, H., 2011. Chemiluminescent reductive acridinium triggering (CRAT)-mechanism and applications. Journal of Analytical Bioanalytical Chemistry, 401(9), 2945-2954

Zozaya-Hinchliffe, M., Martin, D. H. and Ferris, M. J., 2008. Prevalence and abundance of uncultivated *Megasphaera*-like bacteria in the human vaginal environment. Applied and environmental microbiology, 74(5), 1656-1659.

Zundel, M., A, Basturea, G., N. and Deutscher, M., P., 2009. Initiation of ribosome degradation during starvation in *Escherichia coli.* RNA, 15, 977–983

Occurrence of *Pectinatus* and *Megasphaera* in the Major UK Breweries

A. D. Paradh, W. J. Mitchell and A. E. Hill*

ABSTRACT

J. Inst. Brew. 117(4), 498–506, 2011

The occurrence of beer spoilage bacteria belonging to the genera *Pectinatus* and *Megasphaera* in ten major UK breweries was investigated. The sampling points were selected from fermentation areas, beer conditioning areas and beer bottling and canning sites. Multiplex PCR methodology was used for detection of three *Pectinatus* and three *Megasphaera* species using species-specific primers. The presence of six *Lactobacillus* species was also examined. Overall, 117 samples were analysed from ten breweries; six samples were positive for the presence of *Pectinatus* species and three samples were positive for the presence of *Megasphaera* species, while 34 samples were positive for the presence of *Lactobacillus* species. *Lactobacillus* species appeared to be the major potential spoilage microorganisms. Although none of the actual beer samples were found to be positive for *Pectinatus* and *Megasphaera* species, their occurrence in aerobic brewery environments indicates sanitation problems and revealed the presence of highly established biofilms in some breweries.

Key words: beer spoilage bacteria, *Megasphaera,* multiplex PCR, *Pectinatus*

INTRODUCTION

Gram positive lactic acid bacteria of the genera *Lactobacillus* and *Pediococcus*[40] are considered to be the most hazardous beer spoilage bacteria. *Lactobacillus brevis*, *Lactobacillus lindneri* and *Pediococcus damnosus* are reported to be responsible for approximately 70–80% of microbial beer spoilage incidents in Europe during the period 1980 to 2002[2,6]. *L. brevis* has been implicated in more than half of beer spoilage incidents within the same period[3,6,21] while a further 15–20% have been caused by *L. lindneri*[2,6]. *L. coryniformis*, *L. casei and L. plantarum* are other important *Lactobacillus* species which have been reported to spoil beer[6,38] with a frequency of beer spoilage incidents of 3, 2 and 1% respectively[2,47,48]. *Lactobacillus* species cause high turbidity, hazy appearance, unpleasant flavours and a high level of diacetyl in beer[42].

During the 1990s beer spoilage due to Gram negative bacteria belonging to the genera *Pectinatus* and *Megasphaera* increased due to significant reduction of the oxygen content in the final product - a result of improvement in filling technology[18]. However, since then there has been a decrease in spoilage incidences due to these bacteria[2]. *Pectinatus* was reported as a new genus of Gram negative, catalase negative, motile, obligate beer spoilage bacteria in the 1970s, when it was first isolated from a brewery in the United States in unpasteurized beer stored at 30°C[31]. *P. cerevisiiphilus* was later isolated from breweries in Finland, Germany, Norway, Japan, Spain, Netherlands, Sweden and France[15,29,45,50]. In an extensive taxonomic study of anaerobic rods isolated from breweries, a second species of the genus *Pectinatus* was identified as *Pectinatus frisingensis*[44]. *P. frisingensis* differs from *P. cerevisiiphilus* on the basis of growth rate and substrate utilization. In recent studies, a third species, *Pectinatus haikarae* was identified on the basis of 16S rRNA gene sequence analysis and differences in sugar utilization, catalase activity, antibiotic resistance and temperature tolerance compared to the two previously characterised[25]. The growth of *Pectinatus* species is accompanied by extensive turbidity and an offensive aroma similar to rotten egg due to the production of various fatty acids, hydrogen sulphide and methyl mercaptan[15,31].

At present the genus *Megasphaera* is comprised of three brewery associated species. *Megasphaera cerevisiae,* originally described by Engelmann and Weiss[12] was the first brewery associated species, mainly representing low-alcohol beer spoiling cocci. *M. cerevisiae* has been responsible for 3–7% of beer spoilage cases in Europe during the period 1980 to 2002, mainly in non-pasteurised beer[2,3,6]. Later, two novel coccoid shaped bacteria were identified associated with beer spoilage and named *M. paucivorans* and *M. sueciensis*[25]. Spoilage effects of *M. cerevisiae* include turbidity and unpleasant odour, due to production of H_2S and short chain fatty acids. All *Megasphaera* species related to the brewery environment are strictly anaerobic, Gram negative, non-spore forming and non-motile[12,25].

Pectinatus and *Megasphaera* are a major problem from the brewer's point of view as they mainly spoil the beer in the later stages of processing causing financial losses. The contamination causes high turbidity in beer and formation of by-products that cause off-flavours and sour tastes making the beer unsuitable for consumption. This secondary contamination results from ineffective sterilization and pasteurization techniques, hence suitable measures are needed to reduce the incidence of these beer spoilage bacteria. As contamination is caused in the late stages of processing in packaged products, the financial loss is high.

International Centre for Brewing and Distilling, School of Life Sciences, Heriot Watt University, Edinburgh, UK, EH14 4AS.
* Corresponding author. E-mail: A.Hill@hw.ac.uk

Publication no. G-2011-1116-1156

Lactobacillus species are found in almost every stage of the brewing process[21,54]. *Pectinatus* and *Megasphaera* spp. have been reported mainly from spoiled beer and pitching yeast[16]. They have also been isolated from drainage and water pipe systems of beer filling halls, parts of filling machines and the air and floor of filling halls, condensed water on ceilings, loose tiles and in cracks of damaged floors[3,7,32,35]. Plate counting and enrichment remain the principal methods for detection of microbial contamination in breweries during the brewing process and in final products[8]. In recent years, various new methods have been adopted in the brewing industry based on cell and microcolony visualisation and extensive analysis of cellular and genetic content[39,41,46]. PCR based methods have been widely evaluated in brewing laboratories in recent years[1,22–24,27,28,34,37,43,49].

In the current study, multiplex PCR methodology originally described by Asano et al.[1] and later modified by Iijima et al.[23] was used to detect *Pectinatus, Megasphaera* and *Lactobacillus* species. The current literature has no reports on the occurrence of *Pectinatus* and *Megasphaera* species in the UK brewing industry and hence the main objective of this study was to investigate scope and occurrence of these microorganisms in brewery environments in the UK.

MATERIALS AND METHODS

Pure cultures and culture conditions

Species and strains used in this study are shown in Table I. *Pectinatus* and *Megasphaera* were maintained on PYF agar (peptone-yeast extract-fructose)[12] and *Lactobacillus* and *Pediococcus* species were maintained using MRS agar (Oxoid)[10]. Working cultures were obtained by inoculating 10 µL of pure culture onto the specified agar plates and incubating in anaerobic conditions under an atmosphere of N_2:H_2:CO_2 (80:10:10) using a Don Whitley Mac-500 anaerobic cabinet for 4 days at 30°C. A single colony was picked and inoculated into 50 mL of specified broth and incubated as described above.

Sample collection

Based upon information on occurrence and survival sites of the microorganisms in brewery environments, all the sampling points were selected from the fermentation area, conditioning tanks and packaging sites, where anaerobic conditions could prevail or the sites are prone to biofilm formation. A schematic diagram of sample points is shown in Fig. 1. All the samples were taken in the form of sterile swabs, rinse liquor or beer samples.

The pre-reduction of autoclaved medium in aliquots of 62.5 mL in 250 mL bottles was carried out by purging with anaerobic gas mixture N_2:H_2:CO_2 (80:10:10) using Don Whitley Mac 500 anaerobic cabinet followed by incubation of media in anaerobic conditions under an atmos-

Table I. Reference strains of beer spoilage bacteria.

Bacteria	Strains
Pectinatus cerevisiiphilus	ATCC 29359, DSM 20467
Pectinatus frisingensis	VTT E 79100, DSM 6306
Pectinatus haikarae	VTT E 88330, DSM 16980
Megasphaera cerevisiae	ATTC 43254, DSM 20461
Megasphaera sueciensis	DSM 17042
Megasphaera paucivorans	DSM 16981
Lactobacillus brevis	ICBD culture collection strain[a]
Lactobacillus casei	ICBD culture collection strain[a]
Lactobacillis paracollinodes	ICBD culture collection strain[a]
Lactobacillus plantarum	ICBD culture collection strain[a]
Lactobacillus corynformis	ICBD culture collection strain[a]
Pediococcus damnosus	ICBD culture collection strain[a]
Pediococcus inopinatus	ICBD culture collection strain[a]
Pediococcus pentosaceus	ICBD culture collection strain[a]

[a] Culture collection strain from the International Centre for Brewing and Distilling (ICBD), School of Life Sciences, Heriot Watt University, Edinburgh, UK.

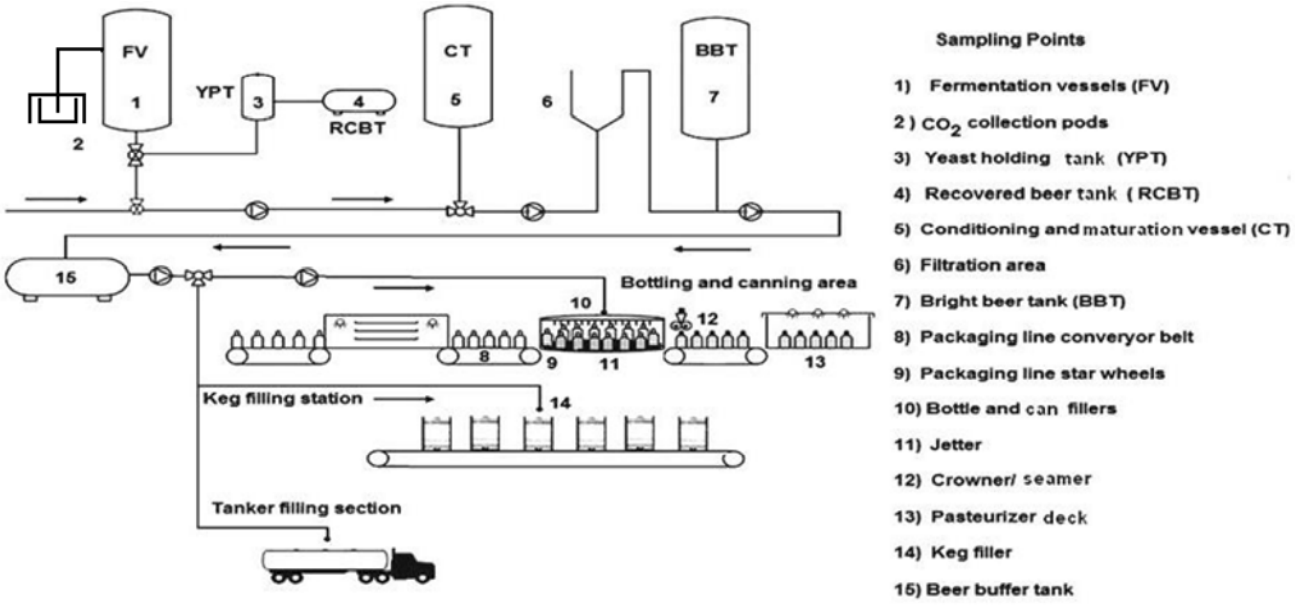


Fig. 1. Schematic representation of sampling points used in the present study.

phere of N_2:H_2:CO_2 (80:10:10) overnight at 30°C. Pasteurised commercial lager containing 4% ABV was degassed in a sterile container by heating at 60°C for 15 min and reduced by purging with anaerobic gas mixture N_2:H_2:CO_2 (80:10:10) using Don Whitley Mac 500 anaerobic cabinet followed by incubation of media in anaerobic conditions under an atmosphere of N_2:H_2:CO_2 (80:10:10) overnight at 30°C.

Swab samples were taken mainly from beer bottling lines and canning lines, specifically from equipment and sites which come into direct contact with packaging materials or finished products. Swabs from bottle conveyor belts, in-feed and outlet star wheel, jetters, crowner and filler tubes were taken. Swabs were taken using sterile swabs and immediately inoculated into a pre-reduced mixture of a 250 mL volume of MRS broth + 1% fructose: pasteurised beer with 4% ABV (1:4) ratio (v/v). Bottles were sealed with parafilm and maintained under anaerobic conditions using an anaerogen kit (Merck) in an anaerobic jar (Merck) at room temperature and transferred to an anaerobic chamber within 12 h for further incubation.

Rinse samples mainly included samples from fillers and wash liquid from fermentation CO_2 collecting pods. Beer samples were selected from fermentation tanks, yeast holding tanks, bright beer tanks and beer buffer tanks. For rinse liquor and direct beer samples, liquid was directly poured into a sterile 250 mL bottle containing 62.5 mL pre-reduced MRS broth +1% fructose. Bottles were sealed with parafilm and maintained under anaerobic conditions using an anaerogen kit (Merck) in an anaerobic jar at room temperature and transferred to an anaerobic chamber within 12 h. All samples were incubated at 30°C for 14 days prior to DNA extraction. Fructose was utilised to enhance the growth of *Megasphaera* species. For samples containing brewing yeast cells, 50 ppm cycloheximide was used to suppress the growth of yeast[26,33].

Cell harvesting and DNA extraction

All the enriched samples were centrifuged at 12,000 rpm for 5 min to concentrate cells. A 500 µL aliquot of concentrated cell suspension was transferred to a 1.5 mL tube and repeatedly washed with sterile deionised water before being used for DNA isolation. Alternatively for some of the samples, 50 µL of concentrated cell suspension was inoculated onto MRS agar + 1% fructose and incubated for 4 days under anaerobic conditions at 30°C and DNA was extracted from representative colonies picked up and resuspended aseptically into 500 µL of sterile deionised water. DNA extraction was carried out using a Qiagen/Gentra-Puregene® kit according to the manufacturer's instructions. Successful DNA extraction was confirmed by running 5 µL of DNA sample on a 1.5% agarose gel.

Primer selection

All the primers were based on rRNA gene sequences and in some species the internal transcribed spacer (ITS) region. The details of primer sequences, target DNA and predicted product sizes are shown in Table II. All primers were purchased from Eurofins MWG Operon (UK). Solution of primers was carried out according to the manufacturer's instructions (to obtain a concentration of 100 pmol/µL) using sterile deionised water and they were then stored at –20°C.

PCR and gel electrophoresis

The multiplex PCR reactions were set up in three reaction formats for each of the three *Pectinatus* species (*Pectinatus* multiplex), three *Megasphaera* species (*Megasphaera* multiplex) and six main beer spoilage *Lactobacillus* species (*Lactobacillus* multiplex), as previously described by Asano et al.[1] and Iijima et al.[23] Certain modifications were made in the multiplex PCR method to ensure specificity and reactivity in order to overcome false positive or false negative results. All three multiplex PCR mixes were specific when checked against closely related species as shown in Table I. The sensitivity of all three PCR multiplexes was determined using serially diluted genomic DNA from target species and positive results were evaluated based on a visible band being obtained on

Table II. List of primers used for detection of *Pectinatus, Megasphaera* and *Lactobacillus* spp. by multiplex PCR[a].

Method	Primer	Direction	Primer sequence (5′ to 3′)	Target species	Target DNA	Product size (bp)
Pectinatus multiplex	16C-F	Forward	CGTATGCAGAGATGCATATT	*P. cerevisiiphilus*	16S-rDNA	621
	IC-R	Reverse	CACTCTTACAAAGTATCTAC	*P. cerevisiiphilus*	ITS region	
	16F-F	Forward	CGTATCCAGAGATGGATATT	*P. frisingensis*	16S-rDNA	701, 883
	IF-R	Reverse	CCATCCTCTTGAAAATCTC	*P. frisingensis*	ITS region	
	Phf1	Forward	AATACCGAATGTTGTAAGAG	*P. haikarae*	16S-rDNA	508
	Phr2	Reverse	CTCTCCTGCACTCAAGACAT	*P. haikarae*	16S-rDNA	
Megasphaera multiplex	mc-f4	Forward	ACCGAATACGATCTAAAG	*M. cerevisiae*	16S-rDNA	452
	mc-rf	Reverse	TTAAGACCGACTTACCGA	*M. cerevisiae*	16S-rDNA	
	Msp-f	Forward	TATGGCCAATACCCATAGAT	*M. sueciensis*	16S-rDNA	155
	Msp-r	Forward	CACTTTTAAGACAGACTTGA	*M. paucivorans*	16S-rDNA	
Lactobacillus multiplex	LBP2	Forward	CTGATTTCAACAATGAAGC	*L. brevis*	16S-rDNA	861
	L74P1	Forward	GGATTTTAACATCGGATGAG	*L. paracollinoides*	16S-rDNA	854
	LCP11	Forward	GAACCGCATGGTTCTTGGC	*L. casei*	16S-rDNA	729
	LOP4	Forward	GGGACTAGAGTAACTGTTAGTCC	*L. corynformis*	16S-rDNA	453
	LPP7	Forward	GTTGTTAAAGAAGAACTTATC	*L. plantarum*	16S-rDNA	490
	LLITSF8	Forward	AACTTACACCGATCAAAATC	*L. lindneri*	ITS region	850
	LL23SR12	Reverse	CTTAACCTTGCATGCAACT	*L. lindneri*	16S-rDNA	-----
	UNP1	Reverse	CCGTCAATTCCTTTGAGTTT	*Lactobacillus* spp. (consensus primer)	23S-rDNA	[a]

[a] Primer UNP1 is shared as a common reverse primers by all five *Lactobacillus* species except *L. lindneri.* Source: Iijima et al.[23]

the agarose gel. Optimization was also necessary to overcome certain variable components such as primer concentration, the nature of the DNA template, quality of Taq polymerase, concentration of the buffer components.

For each reaction mixture 0.5 µL (2.5 units) of BIOTAQ™ DNA Polymerase (BIOLINE) was used. Standard reaction buffer containing a final concentration of 0.8 mM $(NH_4)_2SO_4$, 3.5 mM Tris-HCL, 1.5 mM $MgCl_2$ and 0.2 mM of each of the four dNTPs was used. For *Pectinatus* and *Megasphaera* multiplexes, 1 µL of each primer (100 pmol/µL) was used, for *Lactobacillus* multiplex primer concentrations were as previously described by Asano et al.[1] A 1 µL aliquot of extracted DNA solution was used as a template and the final volume of the reaction mixture was made to 50 µL using sterile deionised water. PCR reactions were performed using BIORAD and Applied Biosystem thermal cyclers. Positive controls were maintained by using a 1 µL DNA template of *P. frisingensis, M. cerevisiae* and *L. brevis* for *Pectinatus, Megasphaera and Lactobacillus* multiplex PCR respectively. Negative controls were maintained using the reaction mixture as described above, but with no DNA template.

The PCR amplification was carried out with an initial denaturation for 4 min at 95°C followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and primer extension at 72°C for 1 min. Final primer extension was carried out for 4 min at 72°C followed by an end hold at 4°C. PCR products were stored at 5–6°C before analysis by gel electrophoresis using 2% agarose gels in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) containing ethidium bromide for DNA staining. A 5 µL aliquot of PCR product was used for analysis and a 100 bp ladder (Hyper ladder IV- BIOLINE) was used as the molecular size marker.

For *Lactobacillus* multiplex, certain similar size amplified fragments were confirmed using simplex PCR as described above, except 1 µL each of species specific primer was used.

RESULTS AND DISCUSSION

Optimization of culture enrichment and multiplex PCR method

Collection and enrichment of samples were important tasks during the study. All the samples were treated on site soon after collection and anaerobic conditions were maintained during transportation of samples to the laboratory by using an anaerogen kit (Merck) and anaerobic jars. PCR is a highly sensitive method for detection of even low levels of contaminants in samples, but for detection of highly anaerobic bacteria, enrichment of samples was carried out for 14 days. For strict anaerobes, culture enrichment is needed to achieve detectable numbers of cells in samples. In addition sometimes the high volume of sample is more important than incubation time to achieve detectable growth of target microorganisms[26] and the volume of samples was 250 mL to overcome this limitation of the enrichment method.

Multiplex PCR methodology was used to detect *Pectinatus*, *Megasphaera* and *Lactobacillus* beer spoilage species, as it was recently used successfully for the comprehensive detection of major beer spoilage bacteria[1,23]. The optimisation of the multiplex PCR method was carried out according to a stepwise protocol described by Henegariu et al.[19] Genomic DNA isolated from pure cultures was used to test Multiplex PCR regimes. Modifications in the multiplex PCR method were made to determine the amplification of weak loci by modifying the primer concentration and optimizing PCR cycles[19]. PCR reactions were optimized and successfully used for further detection of real brewery samples. The original multiplex protocols[1,23] comprised of 30 cycles of denaturation, annealing and extension and the 15 sec, 15 sec and 30 sec respectively, was modified to 30 cycles of 30 sec, 30 sec and 1 min respectively, for all three multiplex PCR methods. It was verified that *Pectinatus*, *Megasphaera and Lactobacillus* species were detected with high specificity and selectivity.

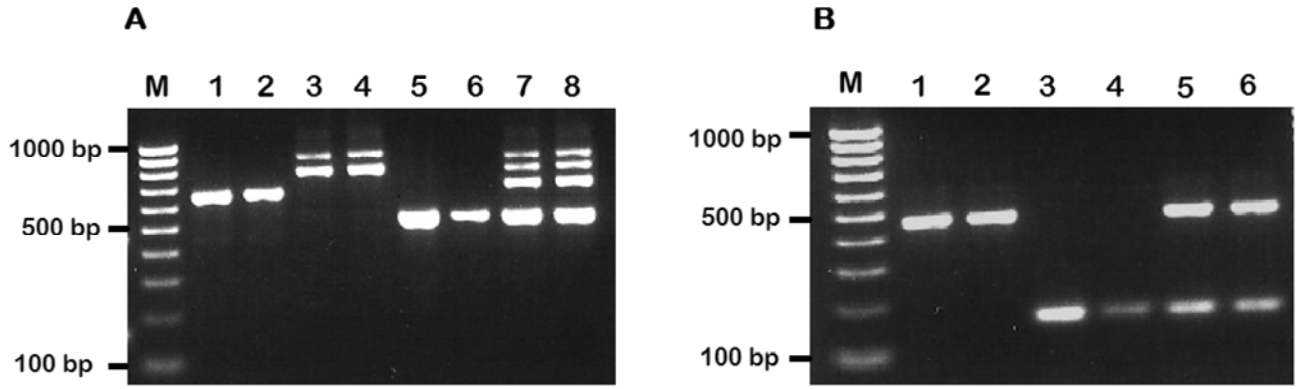


Fig. 2. Specificity of *Pectinatus* and *Megasphaera* multiplex primers was evaluated. **A,** *Pectinatus* multiplex PCR was carried out using different combinations of target bacterial species, 1 and 2 represent *Pectinatus* multiplex results for *P. cerevisiiphilus;* 3 and 4 represent *Pectinatus* multiplex for *P. frisingensis;* 5 and 6 represent *Pectinatus* multiplex for *P. haikarae;* 7 and 8 represent *Pectinatus* multiplex for all three *Pectinatus* species. **B,** *Megasphaera* multiplex PCR was carried out using different combinations of target bacterial species, 1 and 2 represent *Megasphaera* multiplex results for *M. cerevisiae;* 3 and 4 represent *Megasphaera* multiplex for *M. paucivorans;* 5 and 6 represent *Megasphaera* multiplex for *M. cerevisiae* and *M. paucivorans;* M represents 100 bp DNA ladder (Hyper ladder IV Bioline).

Table III. Multiplex PCR results for the brewery samples.

Brewery number		1	2	3	4	5	6	7	8	9	10
Total no. of samples		10	10	10	10	15	15	7	10	10	20
No. of positive samples through multiplex PCR	*P. cerevisiiphilus*	1[a]		1[a]							
	P. frisingensis		2[a]			2[a]					
	P. haikarae										
	M. cerevisiae		1[a]	1[a]							
	M. paucivorans & *M. sueciensis*		1[a]								
	L. brevis	5[ab]	3[ab]	2[a]	1[a]	2[a]	2[a]			1[b]	
	L. lindneri			2	3[a]			1[b]	1[b]	4[ab]	2[a]
	L. casei									1[b]	2[a]
	L. corynformins								1[b]		
	L. plantarum							1[b]			
	L. paracollinoides										

[a] Samples collected from indirect sampling points – swabs and rinse samples from vessels and packaging equipment.
[b] Samples collected from direct beer samples – beer sample/fermenting wort and yeast slurry.

Specificity of *Pectinatus* and *Megasphaera* multiplexes has been illustrated in Fig. 2. All three multiplex PCR were found to be able to detect less than 100 fg of target DNA, where positive results were concluded based on visibility of an amplified band on an agarose gel (data not shown).

Multiplex PCR results

During the investigation of anaerobic beer spoilage bacteria in major UK breweries, 117 samples from ten major breweries were analysed. Of these 117 samples, two samples were positive for *P. cerevisiiphilus,* four samples were positive for the presence of *P. frisingensis,* two samples showed the presence of *M. cerevisiae* and one sample was found positive for the presence of *M. sueciensis* and *M. paucivorans* (detected by the same pair of primers). PCR positive samples for *Pectinatus, Megasphaera* and *Lactobacillus* multiplexes are shown (Table III). *L. brevis* and *L. lindneri* were found to be the most frequently occurring *Lactobacillus* species with 16 and 13 positive samples respectively, while *L. casei, L. plantarum* and *L. corynformis* were found in three, one, and one samples respectively. Ten actual beer samples were positive for the presence of *Lactobacillus* species, mainly from conditioning areas and filtration units.

Pectinatus multiplex PCR samples from star wheels of bottling lines from breweries one and three were positive for the presence of *P. cerevisiiphilus,* while for *P. frisingensis,* two conveyor belt sterile swab samples from brewery two and two samples both from the CO_2 collecting bubble pods of fermenters from brewery five were positive. All six positive samples for *Pectinatus* multiplex were from indirect sampling points and none of the isolates from direct beer samples were found to be positive. It was interesting to find that the samples from the star wheels and conveyor belts, samples which are highly aerobic in nature, showed the presence of strictly anaerobic beer spoilage bacteria. The liquid rinse samples from the CO_2 bubble pods were also of note, as the presence of *Pectinatus* species in the fermentation area is considered to be rare, but the isolation of anaerobic beer spoilage bacteria from CO_2 recovery systems has been frequently reported from the breweries in UK (brewery personal communication). The samples from breweries two and three were each positive for presence of *Megasphaera cerevisiae* and one sample from brewery two taken from the conveyor belt swab of the canning lines was positive for the presence of *M. paucivorans* and *M. sueciensis*. None of the samples from the other breweries (brewery four, six, seven, eight, nine and ten) showed the presence of *Pectinatus* or *Megasphaera* by multiplex PCR. On the other hand *Lactobacillus* species were found to be distributed among the samples from all of the breweries.

Survival of strictly anaerobic bacteria in this aerobic environment can possibly be due to biofilm formation[6,47]. Instruments used in the filling process are prone to formation of biofilms, which are a niche for various beer spoiling microorganisms. The slime produced by these biofilms can protect microbes from routine cleaning procedures. Yeast and *Lactobacillus* species can dwell in these slimes, while the lactic acid produced by *Lactobacillus* species can be metabolized to propionic acid by anaerobic bacteria such as *Pectinatus* species, which can cause undesirable changes to final products[52]. Detection of low levels of *Pectinatus* from biofilms on a conveyor belt in a beer bottling line based on fatty acid profiles has previously been reported[53]. The presence of *Pectinatus* and *Megasphaera* species in fermentation areas and bottling lines of the four major breweries in the UK (breweries one, two, three and five) shows that *Pectinatus* and *Megasphaera* species are natural inhabitants of the breweries in the UK and not infrequent invaders. Breweries one to five were sampled during the months of March to August and breweries six to ten were sampled during the months of September to February. The concentration of *Pectinatus* and *Megasphaera* in brewery environments, in hotter months of the year, could be estimated to be higher than in the cooler months of the year.

In all ten breweries, conventional microbiological practices were adopted for the detection of beer spoilage contaminants based on plate count methods. For the detection of beer spoilage anaerobes, Raka Ray medium has been recommended by European Brewing Convention (EBC)[42] and this medium supplemented with cycloheximide and 2-phenyl ethanol was utilized in all of the ten breweries. In addition, two breweries utilised NBBC broth for the detection of anaerobes. None of the breweries use SMMP medium (Selective Medium for detection of *Megasphaera* and *Pectinatus*)[33] for detection of *Pectinatus* and *Megasphaera* in brewery samples. The Raka Ray medium has the limitation of detecting only facultatively anaerobic bacteria belonging to *Lactobacillus* species and the recov-

Table IV. Summary of hygiene monitoring, inspection and microbial methods utilized in the breweries.

Brewery no.	Capacity (Hl)	Hygiene certification[a]	Packaging facilities	Microbial detection methods/media used for detection of anaerobes	Hygiene inspection	CIP formulation used for packing lines
1	1,900,000	No data	bottling, canning, kegging	Plate count method, Raka Ray	No data	Automatic caustic CIP (1–2%) twice weekly
2	4,000,000	ISO-9001	canning, kegging, casking	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP (1–2%) twice weekly
3	9,000,000	ISO-9001	bottling, canning, kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP (1–2%) twice weekly
4	4,000,000	ISO-9001	bottling, kegging, casking	Plate count method, Raka Ray	No data	Automatic caustic CIP (1–2%) twice weekly
5	4,000,000	ISO-9001	bottling, canning, kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP (1–2%) + combination of para acetic acid (PAA) and chlorine (Cl_2), twice weekly
6	3,800,000	BRC, HACCP	bottling, canning, kegging	Plate count method, Raka Ray, NBBC broth	ATP bio-luminescence	Automatic acid CIP commercial formulation (Johnson Diversey Chemicals, UK)
7	1,900,000	ISO-9001, BRC	kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP (1–2%) after every use
8	1,100,000	ISO-14000, BRC	kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP twice weekly
9	1,100,000	ISO-9001, ISO-22000, ISO-14000	kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP twice weekly
10	No data	BRC	bottling, canning, kegging	Plate count method, Raka Ray, NBBC broth	ATP bio-luminescence	Automatic caustic CIP every 48 h, acid CIP occasionally

[a] ISO: International Organization for Standardization. BRC: British Retail Consortium. HACCP: The Hazard Analysis and Critical Control Point certification.

ery rate on this medium is not good (brewery personal communication), hence it can be confirmed that except NBBC, no effective medium is utilized to specifically detect *Pectinatus* and *Megasphaera* in the UK breweries. The identification of brewery contaminants is mainly based on microscopic analysis. Thus it can be concluded that microbial spoilage due to anaerobic bacteria cannot be specified by the conventional methods used in these breweries unless NBBC is used. The summary of hygiene monitoring and microbial methods adopted in the studied breweries is given in Table IV.

Cleaning and hygiene validation of fermentation tanks, beer storage tanks and packaging lines was carried out by using an ATP bioluminescence method in eight out of the ten breweries. The sensitivity of the ATP method is not suitable for detection of low levels of contaminants; moreover some residues of cleaning agents and disinfectants could affect the enzyme reaction causing light production thus giving non-specific results[30]. ATP bioluminescence is not suitable for the actual detection of contaminants in breweries, as the results are often not similar to those obtained by conventional methods for the same samples[36].

It has been observed that beer with a low alcohol content is more prone to spoilage by *Pectinatus* and *Megasphaera* species. *Pectinatus* species are more resistant to acidic pH and can survive at a pH of 4.1[17]. The pH tolerance of these anaerobic bacteria is influenced by the presence of ethanol[47]. *Pectinatus* and *Megasphaera* species are tolerant to hop bitter substances and can grow in beer with bitterness ranging between 33–38 EBC bitterness[5,29]. *P. frisingensis* shows significant ability to maintain internal homeostasis to mild heat treatment[51] and also its thermal resistance is high compared to *P. cerevisiiphilus*[13]. The growth of *Pectinatus* species is affected significantly by the oxygen content of the beer and has been observed at a dissolved oxygen content of 1.91 mg/L[45]. Modern filling techniques have limited the oxygen content of beer to 0.4–0.8 mg/L, which makes the growth and proliferation of *Pectinatus* in beer possible[9]. The growth of *Megasphaera* in beer with 3.5% ethanol (w/v) is completely restricted[14].

A routine pasteurisation of beer (27–30 PU) is sufficient to inhibit all microorganisms in the beer[4]. *Pectinatus* can be inhibited by a heat treatment of 58–60°C for 1 min, which is less than routine pasteurisation treatment[14]. Aseptic sterilisation using 0.45 μm filters is as effective as flash pasteurisation[4]. It has been reported that *Pectinatus* and *Megasphaera* are susceptible to most of the disinfectants used in the breweries such as iodine, chlorine, and formaldehyde[14]. *Pectinatus* and *Megasphaera* are easy to control via thermal and disinfectant treatment, but these microorganisms still survive in hard to access corners or in biofilms, which are not easy to access and disinfect[47].

In recent years compared to premium lager, there has been development of sub-premium lager brands with a low alcohol content and also mid-strength lager. These brands are at an increased risk from secondary contaminants including *Pectinatus*, primarily due to the low alcohol content of the beer. If these brands are brewed and packaged in the same conditions observed during the current study, the potential risk of contamination in the final packaged product cannot be denied.

The presence of *Pectinatus* species on conveyor belts and star wheels of beer filling lines signifies a higher risk

for packaged beer[18]. *Pectinatus* can be transmitted to fillers and subsequently to packaged beer via aerosols produced during the filling process[11] and cleaning procedures[20,47]. CO_2 recovery systems are never subjected to cleaning regimes as this involves intensive dismantling of equipment (brewery personal communication), hence the bacteria can prevail in this part of the brewery throughout the year, creating a potential threat to packaged beer products in several ways.

The presence of *Pectinatus* and *Megasphaera* in highly aerobic brewery environments can be due to the formation of biofilms and symbiotic associations of microorganisms survive within them[47]. Their presence in highly aerobic conditions provides basic knowledge about the complexity of these biofilms. It is thought that anaerobic bacteria dwell in well established biofilms[6,53]. Contamination could also occur in drainage areas and in floors with defects, areas which are often anaerobic. Even though extensive cleaning procedures are adopted periodically in all of the breweries, the cleaning procedures are not effective enough to completely remove attached biofilms and thus strictly anaerobic beer spoilage bacteria can propagate and be dispersed in packaging plants. The hygiene around the filling machine is also important. The lack of any complaints of spoilage signifies that these secondary contaminants in bottling lines are still in their lag phase of adaptation, due to periodic cleaning regimes ensuring hygienic operating conditions as described by Back in 1994[6]. However ineffective cleaning procedures (as we have found in the breweries sampled), allow the continued presence of these microbes in the filling hall, resulting in their concentration approaching a culmination point. Subsequently some breweries can suffer severely from secondary contaminants without any noticeable prior warning[6]. Other possible reasons that there are no reports on anaerobic bacterial contamination in these breweries could be that most of the premium lagers (5% ABV) brewed do not support the growth of *Pectinatus* and *Megasphaera*. However, the presence of these anaerobic beer spoilers in aerobic brewery environments means that there is a very real risk of contamination of unpasteurised or flash pasteurised beers with a low alcohol content.

At present, automated CIP (Cleaning in Place) with a varying concentration of sodium hydroxide (NaOH; 1–2%); cold and hot CIP, once or twice a week is utilised in most breweries. In general, filling equipment is cleaned using automated caustic CIP and foam cleaning after every use. Brewery six utilizes an acid based commercial formulation (Johnson Diversey Chemicals, UK); this brewery showed comparably better hygienic conditions in the brewery equipment and canning lines and none of the samples were positive for *Pectinatus* and *Megasphaera,* and only two samples were positive for *Lactobacillus*.

Brewery five utilizes disinfectants, such as PAA (paraacetic acid) and Cl_2 (chlorine) in addition to caustic CIP, for the cleaning of bottling and canning lines respectively. In some breweries, the practice of increasing caustic concentration (1.5–4%) along with increased temperature in hot CIP is also employed in cases of severe problems of secondary contaminants, but this practice seems to be unnecessary as there is a need for a modification in detergent formulation rather than using a high concentration of caustic CIP, which could be cost intensive.

It may be concluded that alternation in caustic CIP with the use of modified detergent formulations can be beneficial to achieve satisfactory hygiene conditions in breweries and packaging facilities. There is scope for development of modified detergent formulations, as the trend in shifting caustic CIP to alternate formulations can be seen in major lager breweries in the UK.

CONCLUSIONS

This study found the presence of *Pectinatus* and *Megasphaera* spp. from indirect sampling points in four out of ten breweries. Although none of the direct beer samples were found to test positive for anaerobic beer spoilage bacteria, the presence of *Lactobacillus* species in direct beer samples indicates sanitation problems in these breweries. The record of anaerobic microbes and their sampling sites can provide beneficial data for further studies and the experimental results are useful in designing improvements in the UK breweries.

ACKNOWLEDGEMENTS

This project was funded by the Institute of Brewing and Distilling Grants committee and International Centre for Brewing and Distilling, School of Life Sciences, Heriot Watt University, Edinburgh.

REFERENCES

1. Asano, S., Suzuki, K., Kuriyama, H., Yamashita, H. and Kitagawa Y., Application of multiplex PCR to the detection of beer spoilage bacteria. *J. Am. Soc. Brew. Chem.*, 2008, **66,** 37-42.
2. Back, W., Biofilme in der Brauerei und Getränkeindustrie –15 Jahre Praxiserfahrung. *Brauwelt Online,* 2003, **24/25,** 1-5.
3. Back, W., Breu, S. and Weigand, C., Infektionsursachen im Jahre 1987. *Brauwelt,* 1988, **178,** 1358-1362.
4. Back, W., Leibhard, M. and Bohak, I., Flash pasteurization - membrane filtration. Comparative biological safety. *Brauwelt Int.,* 1992, **1,** 42-49.
5. Back, W., Nachweis und Identifizierung Gram Negativer Bierschädlicher Bacterien. *Brauwissenschaft,* 1981, **34,** 197-204.
6. Back, W., Secondary contamination in filling area, *Brauwelt Int.,* 1994, **4,** 326-333.
7. Back, W., Winery. In: Colour Atlas and Handbook of Beverage Biology, W. Back, Ed., Verlag Hans Carl: Nürnberg, Germany, 2005, pp. 113-135.
8. Campbell, I., Microbiological methods in brewing analysis. In: Brewing Microbiology, 3rd edition, F. G. Priest and I. Campbell, Eds., Kluwer Academic/Plenum Publisher: New York, 2003, pp. 367-392.
9. Chowdhury, I., Watier, D. and Hornez, J. P., Variability in survival of *Pectinatus cerevisiiphilus,* strictly anaerobic bacteria, under different oxygen conditions. *Anaerobe,* 1995, **1,** 151-156.
10. De Man, J. C., Rogosa, M. and Sharpe, M. E., A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.,* 1960, **23,** 130-135.
11. Dürr, P., Luftkeimindikation Bierschadlicher Bakterien: Neue Methode mittlels Luftkeimsammelgerat und Luftkeimindikator. *Brauwelt,* 1983, **123,** 1652-1655.
12. Engelmann, U. and Weiss, N., *Megasphaera cerevisiae* sp. nov.: a new Gram negative obligately anaerobic coccus isolated from spoiled beer. *J. Syst. Appl. Microbiol.,* 1985, **6,** 287-290.

13. Flahaut, S., Teirny, Y., Hornez, J. P., and Jeanfils, J., Impact of thermal variation on biochemical and physiological traits in *Pectinatus* species. *Int. J. Food Microbiol.,* 2000, **55,** 53-61.
14. Haikara, A. and Helander, I., *Pectinatus, Megasphaera* and *Zymophilus*. In: The Prokaryotes Vol. 4, M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer and E. Stackebrandt, Eds., Springer-Verlag: New York, 2006, pp. 965-981.
15. Haikara, A., Enari T. M., Lountmaa, K., The genus *Pectinatus,* a new group of anaerobic beer spoilage bacteria. Proceedings of the European Brewery Convention Congress, Copenhagen, IRL press: Oxford, 1981, pp. 229-240.
16. Haikara, A., Invasion of anaerobic bacteria into pitching yeast. Proceedings of the European Brewery Convention Congress, Zurich, IRL press: Oxford, 1989, pp. 537-544.
17. Haikara, A., Lounatmaa, K., Characterization of *Megasphaera* sp., a new anaerobic beer spoilage coccus. Proceedings of the European Brewery Convention Congress, Madrid, IRL Press: Oxford, 1987, pp. 473-480.
18. Haikara, A., The genera *Pectinatus* and *Megasphaera*. In: The Prokaryotes, A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K-H. Schleifer, Eds. Springer Verlag: New York, 2003, pp. 1993-2004.
19. Henegariu, O., Heerema, N. A., Dlouhy, S. R., Vance, G. H. and Vogt, P. H., Multiplex PCR: Critical Parameter and step-by-step Protocol. *Biotechniques,* 1997, **23,** 504-511.
20. Holah, J. T., Industrial monitoring: Hygiene in food processing. In: Biofilms - Science and Technology. L. F. Melo, T. R. Bott, M. Fletcher and B. Capdeville, Eds., Kluwer Publication: Dordrecht, 1992, pp. 645-659.
21. Hollerová, I. and Kubizniaková, P., Monitoring Gram positive bacterial contamination in Czech breweries. *J. Inst. Brew.,* 2001, **107,** 355-358.
22. Homann, F., Bremenr, Z. and Moller-Hergt, G., Lightcycler™ PCR establishing itself as a rapid detection method. Beer spoilage bacteria in microbiological routine analysis, *Brauwelt Int.,* 2002, **5,** 292-296.
23. Iijima, K., Asano, S., Suzuki, K., Ogata, T. and Kitagawa, Y., Modified Multiplex PCR method for comprehensive detection of *Pectinatus* and beer spoilage cocci, *Biosci. Biotechnol. Biochem.,* 2008, **72,** 2764-2766.
24. Juvonen, R. and Haikara, A., Amplification facilitators and preprocessing methods for PCR detection of strictly anaerobic beer-spoilage bacteria of the class Clostridium in brewery samples, *J. Inst. Brew.,* 2009, **115,** 167-176.
25. Juvonen, R. and Suihho, M. L., *Megasphaera paucivorans* sp. nov., *Megasphaera sueciensis* sp. nov. and *Pectinatus haikarae* sp. nov., isolated from brewery samples, emended description of genus *Pectinatus*, *Int. J. Syst. Evol. Microbiol.,* 2006, **56,** 695-702.
26. Juvonen, R., Koivula, T. and Haikara, A., Group-specific PCR-RFLP and real time PCR methods for detection and tentative discrimination of strictly anaerobic beer spoilage bacteria of class Clostridia. *Int. J. Food Microbiol.,* 2008, **125,** 162-169.
27. Juvonen, R., Satokari, R., Mallison, K. and Haikara, A., Detection of spoilage bacteria in beer by PCR. *J. Am. Soc. Brew. Chem.,* 1999, **57,** 99-103.
28. Kiehne, M., Gronewald, C. and Chevalier, F., Detection and identification of beer spoilage bacteria by real time polymerase chain reaction. *Tech. Q. Master Brew. Assoc. Am.,* 2005, **42,** 214-218.
29. Kirchner, G., Lurz, R. and Matsuzawa, K., Biertrübungen durch Bakterien der Gattung Bacteroides. *Monatsschr. Brauwiss.,* 1980, **33,** 461-467.
30. Lappalainen, J., Loikkanen, S., Havana, M., Karp, M., Sjoberg, A. M. and Wietanin, G., Microbial testing methods for detection of residual cleaning agents and disinfectants - prevention of ATP bioluminescence measurements in the food industry. *J. Food Protect.,* 2000, **63,** 210-215.
31. Lee, S. Y., Mabee, M. S. and Jangaard, N. O., *Pectinatus*, a new genus in family *Bacteriodaceae*. *Int. J. Syst. Bacteriol.,* 1978, **28,** 582-594.
32. Lee, S., Y., Mabee, M., S., Jangaard, N. O. and Horiuchi, E. K., *Pectinatus,* a new genus of bacteria capable of growth in hopped beer. *J. Inst. Brew.,* 1980, **86,** 28-30.
33. Lee, S.Y., SMMP-a medium for selective isolation of *Megasphaera* and *Pectinatus* from brewery. *J. Am. Soc. Brew. Chem.,* 1994, **63,** 115-119.
34. Motoyama, Y. and Ogata, T., Detection of Pectinatus spp. by using 16S-23S r-DNA spacer region. *J. Am. Soc. Brew. Chem.,* 2000, **58,** 4-7.
35. Motoyama, Y., The study on strictly anaerobic bacteria capable of spoiling beer. Ph.D. Thesis, Tokyo University: Tokyo, Japan, 2003.
36. Odebrecht, E., Schimidt, H. J. and Franco, B. G. M., Studies on applicability of bioluminescence in the brewery. Comparative studies and critical evaluation. *Brauwelt*, 2000, **140,** 1904-1905, 1908-1915.
37. Ohnishi, A., Abe, S., Nashirozawa, S., Shimada, S., Fujimoto.,N and Suzuki M., Development of a 16S rDNA primer and PCR-RFLP method for the rapid detection of genus *Megasphaera* and species level identification, *J. Appl. Environ. Microbiol.,* 2011, **77,** 5533-5535.
38. Priest, F. G., Gram-positive brewery bacteria. In: Brewing Microbiology. Elsevier: London, 1996, pp. 127-161.
39. Priest, F. G., Rapid identification of microorganisms, In: Brewing Microbiology, 3rd edition, F. G. Priest and I. Campbell, Eds., Kluwer Academic/Plenum Publishers: New York, 2003, pp. 305-328.
40. Rainbow, C., Beer spoilage microorganisms, In: Brewing Science, J. R. A. Pollock, Ed., Academic Press: London, 1981, pp. 491-550.
41. Russell, I. and Stewart, R., Rapid detection and identification of microbial spoilage , In: Brewing Microbiology, 3rd edition, F.G. Priest and I., Campbell, I., Eds., Kluwer Academic/Plenum Publisher: New York, 2003, pp. 267-307.
42. Sakamoto, K., and Konings, W., N., Beer spoilage bacteria and hop resistance, *Int. J. Food Microbiol.,* 2003, **89,** 105-124.
43. Satokari, R., Juvonen, R., Mallison, K., von Wright, A. and Haikara, A., Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by polymerase chain reaction and colorimetric microplate hybridization, *Int. J. Food Microbiol.*, 1998, **45,** 119-127.
44. Schleifer, K. H., Leutritz, M., Weiss, N., Ludwig,W., Kirchhof, G. and Seidel-Rufer, H., Taxonomic study of anaerobic, gram-negative rod shaped bacteria from breweries: Emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingensis* sp. nov., *Selenomonas lacticifex* sp. nov., *Zymophilus raffinosivorans* gen., sp. nov., and *Zymophilus paucivorans*. *Int. J. Syst. Bacteriol.,* 1990, **40,** 19-27.
45. Soberka, R., Sciazko, D. and Warzecha, A., *Pectinatus* nouvelle bactérie pouvant affecter la stabilité biologique du moüt et de la biére. *Bioscience,* 1988, **19,** 31-37.
46. Strogårds, E., Process hygiene control in beer production and dispensing. Espoo 2000. VTT Publications 410: Technical Research Centre Finland, 2000, 105 p.
47. Suzuki, K., 125th Anniversary review: Microbiological instability of beer caused by spoilage bacteria. *J. Inst. Brew.,* 2011, **117,** 131-155.
48. Suzuki, K., Asano, S., Iijima, K. and Kitamoto, K., Sake and beer spoilage lactic acid bacteria – A review. *J. Inst. Brew.,* 2008, **114,** 209-223.
49. Suzuki, K., Iijima, K., Sakamoto, K., Sami, M. and Yamashita, H., A review of hop resistance in beer spoilage lactic acid bacteria. *J. Inst. Brew.,* 2006, **112,** 173-191.
50. Takahashi, N., Presumed *Pectinatus* strain isolated from Japanese beer. *Bull. Brew. Sci.,* 1983, **28,** 11-14.
51. Tholozan, J. L. and Jacquemont, J., Physiological response of *Pectinatus frisingensis,* a beer spoilage bacterium, to mild heat treatments. *Can. J. Microbiol.,* 1999, **45,** 598-606.
52. Tholozan, J. L., Membre, J. M. and Grivet, J. P., Physiology and development of *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis,* two strict anaerobic beer spoilage bacteria. *Int. J. Food Microbiol.,* 1997, **35,** 29-39.

53. Timke, M., Wolking, D., Wang-Lieu, W. Q., Altendorf, K. and Lipski, K., Microbial composition of biofilms in a brewery investigated by fatty acid analysis, fluorescence in situ hybridisation and isolation techniques. *J. Appl. Microbiol. Biotechnol.,* 2005, **66,** 100-107.

54. Vaughan, A., O'Sullivan, T. and Sinderen, D. V., Enhancing microbiological stability of malt and beer. *J. Inst. Brew.,* 2005, **111,** 355-371.

(Manuscript accepted for publication August 2011)